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THE LARVAL GENERA AND SPECIES OF 'HARVEST MITES' (ACARINA: TROMBICULIDAE)

BY CHARLES D. RADFORD

Membre Correspondant, Muséum National d'Histoire Naturelle, Paris

(With 108 Figures in the Text)

INTRODUCTION

The mites of the subfamily Trombiculinae are of special importance since, in the larval form, they are reported from many countries to be a nuisance to human beings by the intense irritation caused by their bites. At least two species of these larvae are the acknowledged vectors of scrub typhus (OXK typhus, 'tsutsugamushi' or Japanese river fever). The nymph and adult mites are free living and are known to be predatory, feeding upon the collembola and also on the eggs of ants, mosquitoes and collembola. In the larval stage they are found as parasites on mammals, birds, snakes, lizards and frogs. They have also been recorded upon a scorpion and upon three families of grasshoppers.

These larval mites are known by various names—in the British Isles as 'harvest mites' owing to their prevalence at this time of the year; in the United States they are called 'chiggers'; in France they are known as 'rouget' and in other countries by still other local names.

Many of these mites are not known in their adult stage, and it has therefore been found expedient to establish genera and species which are based upon the larval characters. It is recognized that this will, at a later date, necessitate the suppression of some of these names when further successful rearing experiments will make it possible to correlate the known larvae with their nymphal and/or adult forms. This can be achieved only by the careful isolation of individual (engorged) larvae in order to establish definitely that the resultant nymph is from that specimen. Too often in the past have we found that a single cluster of larvae taken on a host, though to all appearances being identical, has, upon examination proved to contain more than one species—bulk rearing is, therefore, almost useless.

Breeding experiments, which had been conducted mainly by the Japanese workers, Nagayo *et al.* (1919–21), Kawamura & Yamaguchi (1921) and later by Walch (1923) in Malaya, had not always been successful. They claimed to have successfully reared these mites through their life cycles and gave figures of the nymphs and adults of several species which are indigenous to the Japanese islands. In these papers they stated that the nymphs and adults lived upon plant juices, citing a reed (*Imperata arundinacea* Cyr.) and a daisy (*Artemisia vulgaris* L.).

In 1944–5 the author made several attempts to rear these mites and study their life histories, but with limited success. Fully engorged larvae were placed in cells containing soil in order to approximate as closely as possible the natural biotope of these larvae. In some instances nymphs were later observed, and it was possible

to confirm the work of Walch who had been working in Malaya with similar species, or to add to the number of nymphal stages that could be correlated with their known larvae. In no case was it possible for the author to rear adults from these engorged larvae—the nymphs would survive for a few days then die. In the light of subsequent work carried out by colleagues in the United States it seems that the cause of the failures were either cannibalism amongst the nymphs or the absence of suitable food.

Working therefore from the other angle, soil from the burrows of rats (*Bandicota malabarica* Shaw) was examined by flotation in water. Several mites were obtained which, upon examination were found to be similar to the nymphs but larger and possessed mature sexual organs. These adults were then placed in individual rearing cells and later produced larvae, which, when mounted in P.V.A. and examined under the microscope were found to be the larval *Leptotrombidium deliensis* (Walch, 1923). In the case of *Euschongastia indica* (Hirst, 1915) the adults were found in the soil at the tops of palm trees, where rats had been feeding (Radford, 1946*b*).

Wharton (1946) succeeded in rearing *E. indica* throughout its life cycle and showed that the nymphs fed upon collembola and the eggs of mosquitoes. Experiments carried out in the United States by Jenkins (1947) have shown that certain species of these mites will, in the adult stage, consume as many as fifteen eggs of the mosquito *Aëdes aegypti* in 90 min. Attempts to feed adults of *Leptotrombidium* upon the eggs of this mosquito failed, whereas they would readily feed on the eggs of *Culex*.

What chemical difference is there between the eggs of *Aëdes* and *Culex* that is discernible to these adult Trombiculid mites that they show a preference to one and yet shun the other? Is it possible that the dermal layer of the one is so much tougher that the mite cannot penetrate it or is there some other explanation?

It was stated (Radford, 1946*a*) that of 200 larval species the adults of only some twelve were known. Recent studies and improved breeding techniques has added considerably to the number of adults and nymphs that have now been correlated with their known larvae.

Though the larval stage of *Neotrombicula autumnalis* was first described in 1790 by Shaw, the nymph was described and figured only in 1915 by Hirst, and the adult by André (1929). *Leptotrombidium deliensis* (Walsh, 1923), in its adult form, was only recently described (Radford, 1946*a*).

Prior to the war of 1939–45 these larval Trombiculid mites had received but little attention. Cases of an unknown fever were reported periodically from the rubber plantations of Malaya, India, Burma and the islands of the China Sea, but mortality rates were not high and occurred mainly amongst the coolie labourers. With the outbreak of war in the Far East and Pacific, necessitating the movement of troops through the jungle undergrowth, the important role played by these larval mites in spreading this disease at once received fresh consideration. Outbreaks of scrub typhus were reported amongst Allied forces operating in the jungles of South-East Asia, South-West Pacific and in Northern Queensland. This focused the attention of the Medical Directorates of the Armed Services upon the problem,

and led to the formation of research teams to investigate the disease and the role played by these mites.

The Australian Army had teams working on this problem in New Guinea and Northern Queensland. The U.S. Typhus Commission sent investigators to New Guinea and the islands of the South-West Pacific from which outbreaks of scrub typhus had been reported.

An unusually large outbreak of this disease occurred in an East African brigade engaged in jungle exercises around Embilipitiya, in the south of the island of Ceylon. A team of workers were sent out from the War Office to investigate the disease in Ceylon, the Maldive Islands and Chagos Archipelago in the Indian Ocean, Manipur State on the Assam/Indian border and later in Burma. This team was at first a War Office set-up, part Royal Air Force part R.A.M.C., but was later based at Imphal under the Medical Research Council and included civilian scientists. A team of workers of the U.S. Typhus Commission were later sent to Burma and based at Myitkyina. Much useful information was obtained as a result of these various investigations.

In the post-war period teams have been busy studying these larval mites in Korea and Japan under the auspices of the U.S. Army acting in co-operation with a number of Japanese acarologists. The Scrub Typhus Research Unit, working under the auspices of the Colonial Medical Research Committee, have been based at Kuala Lumpur. Work on these mites has also been carried out in the Belgian Congo and in South Africa. Through the activities of these various teams we have accumulated a vast amount of information on these mites, both as larvae and as nymphs and adults. A number of these projects have, of course, been tied up with investigations into effective repellants and the prophylaxis or treatment of the disease.

In the period 1939–52 various taxonomic problems have arisen which have necessitated the transference of a number of species to other genera. To achieve uniformity in describing new genera and species the American workers collaborated in the publication of a paper giving the terminology which was considered best to describe these mites. In the past, descriptions of new genera and species have been published in various journals—medical, zoological, entomological, veterinary—in the publications of local scientific societies—in a variety of languages—English, French, German, Portuguese, Dutch and Japanese.

The average worker has not ready access to a library containing all, or even most, of these scattered papers, many of which are out of print. The present paper has been prepared to meet the demands of the many workers who have recently, or are at present engaged in Trombiculid surveys and to whom the older literature is inaccessible.

The author, for his own use, commenced making a collection of drawings of the dorsal scuta of all those species in his private collection, or to which he had access, to serve as a quick check when examining material assumed to be new. The figures of these scutae are all drawn to the same scale and are here presented for benefit of workers, to whom many of these species are undoubtedly new. To have given figures for all the known species would have been impossible, for in no collection in

the world are there representatives of all the known species, some having been described from single specimens, in other cases the original specimen has been lost, or was in such bad state as to be useless for illustrating.

I wish to thank my many colleagues for the copies of their publications and the numerous paratype or duplicate slides which they have presented to me. I have, wherever possible, made a distribution of paratype slides to these workers and also to a number of National Museums throughout the world to facilitate the work of others engaged in this interesting study.

Family TROMBICULIDAE Ewing, 1944

Subfamily TROMBICULINAE Ewing, 1929

Genus **Trombicula** Berlese, 1905

T. ablephara Womersley, 1952	*Ablepharus boutoni*
T. abyssinica Radford, 1946 (Fig. 1)	*Vidua fischeri* (Reichenow, 1882)
T. alaskensis Brennan & Wharton, 1950	*Hylocichla ustulata*
T. aplodontiae Brennan, 1946 (Fig. 41)	*Aplodontia r. rufa* Rafinesque, 1817
T. avis Floch & Abonnenc, 1941	*Turdus* sp.
T. agamae André, 1929	*Agama stellio* L. 1758
T. bakeri Ewing, 1946	?
T. batui Philip & Traub, 1950	*Eonycteris spelaea* (Dobson, 1871)
T. biops Wharton, 1948	*Phyllotis boliviensis* Waterhouse, 1846
	P. darwini Waterhouse, 1837
T. bisignata Ewing, 1929 (Fig. 3)	*Microtus p. pennsylvanicus* Ord, 1815
T. blarinae Ewing, 1931	*Blarina brevicauda brevicauda* Say, 1823
T. bodensis Gunther, 1940 (Fig. 9)	*Tragulus borneanus*
T. brevitarsa Radford, 1953 (Fig. 28)	*Rhinolophus clivosus acrotis* Heuglin, 1861
T. buloloensis Gunther, 1939	*Echymipera cockerelli* Ramsay, 1877
	Megapodius r. reinwardt Dumont, 1823
	Talegalla j. jobiensis Meyer, 1874
	Casaurius casaurius L. 1758
T. buxtoni Womersley, 1952	*Rattus* sp.
T. canis Floch & Abonnenc, 1941	*Homo sapiens* L.
T. cervulicola Ewing, 1931	*Muntiacus muntjak aureus* Smith, 1826
T. chara Wharton, 1948	*Phyllotis boliviensis* Waterhouse, 1846
	P. darwini Waterhouse, 1837
T. chiroptera Womersley, 1943	Bats
T. claviglia Radford, 1948 (Fig. 12)	*Graphiurus* (*Claviglis*) *murinus* ssp.
T. claviglicola Lawrence, 1949	*Claviglis m. murinus* (Desmarest, 1822)
T. corvi Kawamura & Yamaguchi, 1921	*Gallus* sp.
T. cynictia Radford, 1942 (Fig. 38)	*Cynictis penicillata ogilbyi* Smith, 1834
T. cynos Ewing, 1937	*Procyon lotor* L.
T. dasyproctae Ewing, 1937	*Dasyprocta p. punctata* Gray, 1842
T. densipiliata Walch, 1923	*Rattus* sp.
T. eptesici Brennan, 1947 (Fig. 39)	*Eptesicus fuscus pallidus* Young, 1908
T. fahrenholzi Oudemans, 1910	*Miniopteris schreibersi* Kuhl, 1817
T. filamentosa Radford, 1953	*Rhinolophus clivosus acrotis* Heuglin, 1861
T. fletcheri Womersley, 1943	*Rattus rattus* ssp.
T. frittsi Wharton, 1945	*Gehyra oceanica* Gray, 1845
	Varanus indicus Daudin, 1802
	Rattus leucopus praetor Thomas, 1888
T. guineense Bruyant & Joyeaux, 1913	*Gallus domesticus* L. 1758
	Erythrocebus patas pyrrhonotus Hombron & Ehrenberg, 1838
	Cercopithecus aethiops sabaeus L. 1758

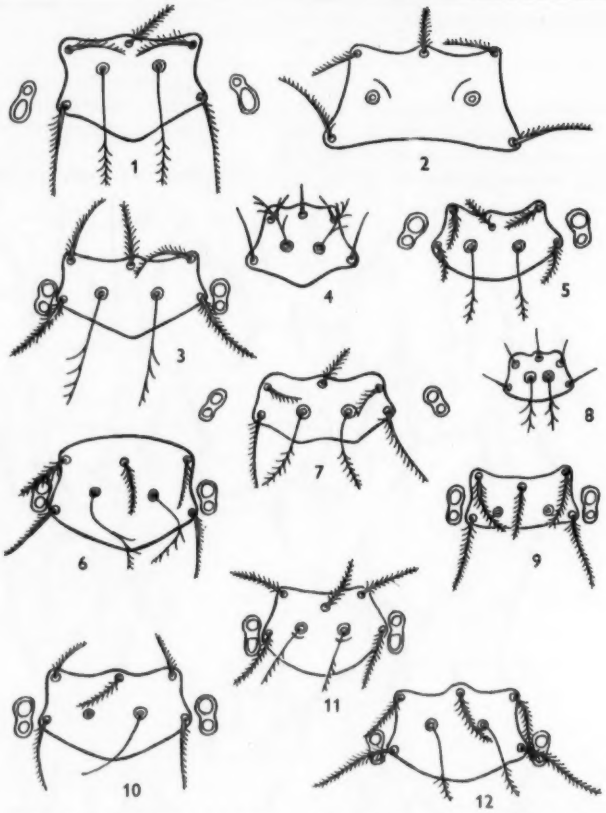


Fig. 1. *Trombicula abyssinica* Radford, 1946.
Fig. 2. *T. praomyia* Radford, 1942.
Fig. 3. *T. bisignata* Ewing, 1929.
Fig. 4. *T. mastomyia* Radford, 1942.
Fig. 5. *T. montanensis* Brennan, 1946.
Fig. 6. *T. minor* Berlese, 1905.
Fig. 7. *T. hamertoni* Radford, 1942.
Fig. 8. *T. munda* Gater, 1932.
Fig. 9. *T. bodensis* Gunther, 1940.
Fig. 10. *T. samboni* Womersley, 1939.

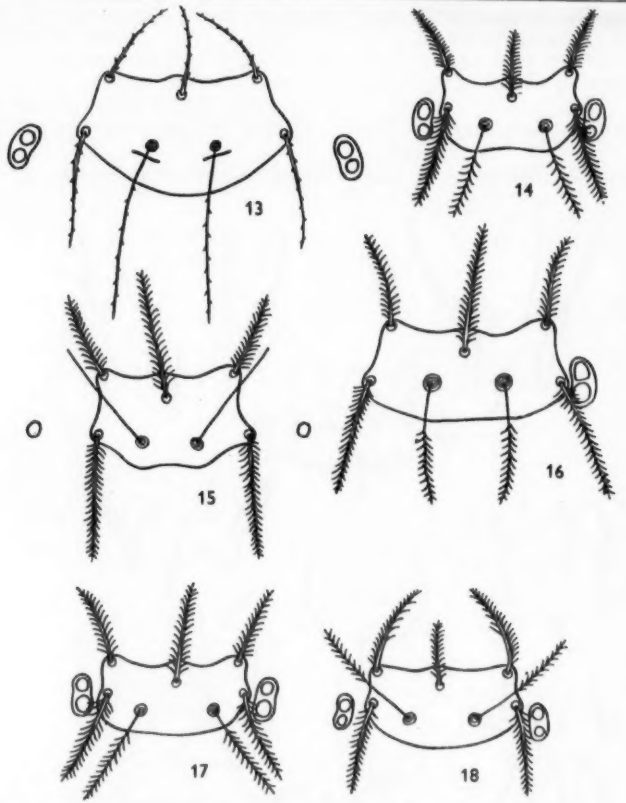
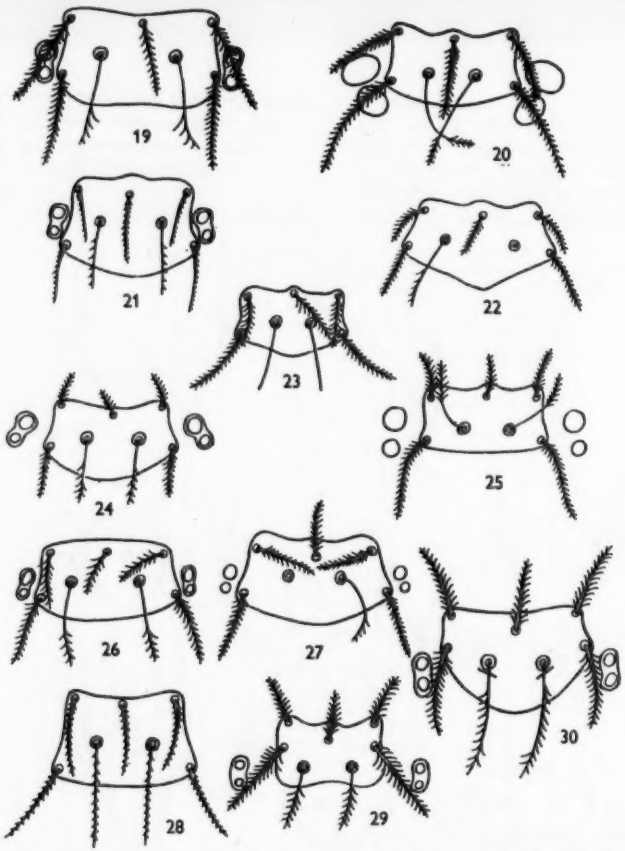


Fig. 11. *T. keiterti* Fuller & Philip, 1950.
Fig. 12. *T. claviglia* Radford, 1948.
Fig. 13. *Trombicula pomeranzevi* Shluger, 1948.
Fig. 14. *T. orientalis* Shluger, 1948.
Fig. 15. *T. miyajimai* Fukuzumi & Obata, 1951
Fig. 16. *T. miyazakii* Sasa.
Fig. 17. *Lepto-trombidium pallida* Nagayo, 1919.
Fig. 18. *L. palpalis* Nagayo, 1919.



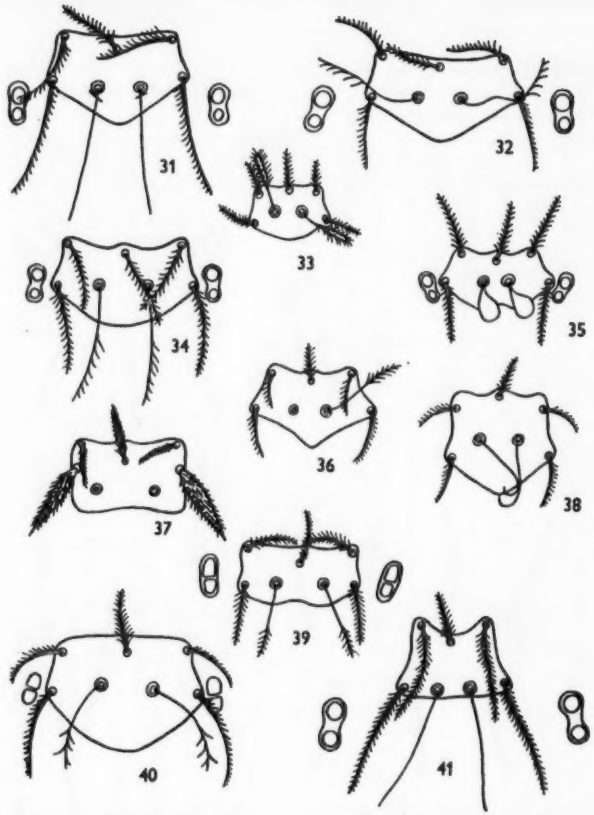


Fig. 19. *Eutrombicula rioi* (Gunther, 1939).
Fig. 20. *E. anous* (Wharton, 1945).
Fig. 21. *E. hirsti* (Sambon, 1928).
Fig. 22. *E. splendens* (Ewing, 1913).
Fig. 23. *E. batatus* (L. 1758).
Fig. 24. *E. alfreddugesii* (Oudemans, 1910).
Fig. 25. *E. pluvius* (Wharton, 1945).
Fig. 26. *E. gouldii* (Oudemans, 1926).
Fig. 27. *E. hakei* (Radford, 1946).
Fig. 28. *Trombicula brevitarsa* Radford, 1952.
Fig. 29. *Leptotrombidium fuji* Kuwato *et al.* 1950.
Fig. 30. *Tragardhula tamiyai* (Philip & Fuller, 1950).
Fig. 31. *Neotrombicula whartoni* Ewing, 1929.
Fig. 32. *N. harperi* (Ewing, 1928).
Fig. 33. *N. spicea* (Gater, 1932).
Fig. 34. *N. microti* (Ewing, 1928).
Fig. 35. *Schöngastia philipi* Womersley & Kohls, 1947.
Fig. 36. *Tragardhula ilesi* (Radford, 1948).
Fig. 37. *Neotrombicula hastata* (Gater, 1932).
Fig. 38. *Trombicula cynictia* Radford, 1942.
Fig. 39. *T. eptesici* Brennan, 1947.
Fig. 40. *Neotrombicula sylvilagi* Brennan & Wharton, 1950.
Fig. 41. *Trombicula aplodontiae* Brennan, 1946.

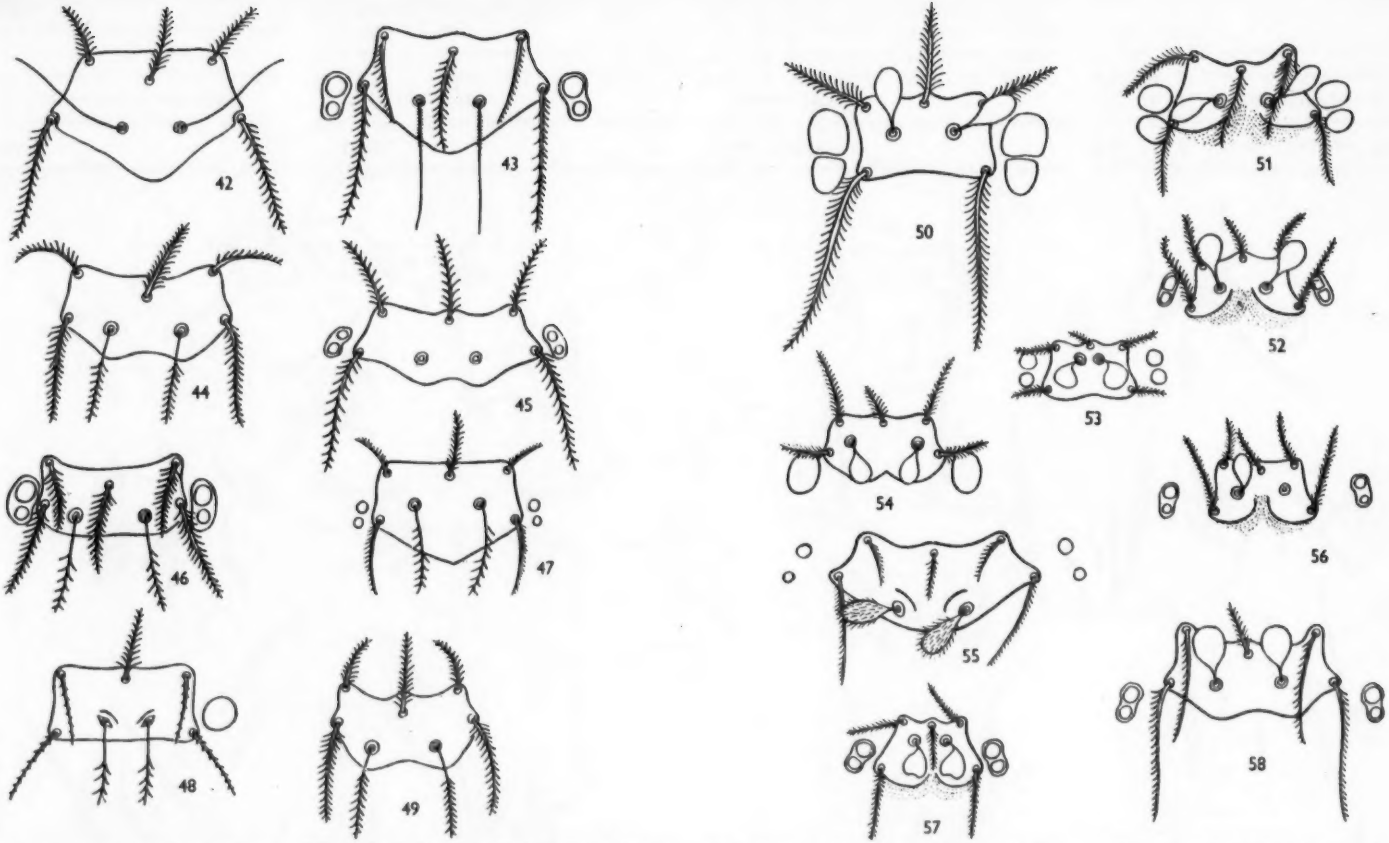


Fig. 42. *Neotrombicula dinehartae* Brennan & Wharton, 1950.
Fig. 43. *N. jewetti* Brennan & Wharton, 1950.
Fig. 44. *Leptotrombidium intermedia* (Nagayo *et al.* 1920).
Fig. 45. *Trombicula jamesoni* Brennan, 1948.
Fig. 46. *T. kitasatoi* Fukuzumi & Obata, 1951.
Fig. 47. *T. parmifera* Womersley, 1952.
Fig. 48. *T. samara* Radford, 1952.
Fig. 49. *T. tanaka-ryoi* Kawashima & Sasa, 1952.
Fig. 50. *Neoschöngastia carveri* Wharton, 1946.
Fig. 51. *N. bougainvillensis* Wharton, 1946.
Fig. 52. *N. americana solomonis* Wharton, 1946.
Fig. 53. *N. atollensis* Wharton, 1946.
Fig. 54. *N. egretta* Wharton, 1946.
Fig. 55. *Euschöngastia guntheri* (Radford, 1942).
Fig. 56. *Neoschöngastia americana* (Hirst, 1921).
Fig. 57. *N. thomasi* (Radford, 1946).
Fig. 58. *Euschöngastia aethomyia* (Radford, 1942).

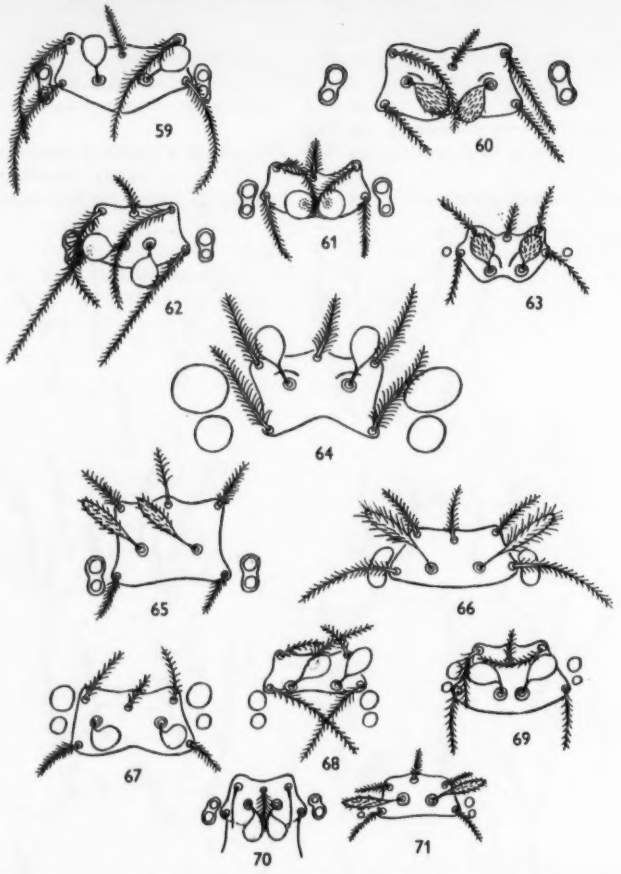


Fig. 59. *Euschöngastia longispina* (Radford, 1948).
Fig. 60. *E. yeomansi* (Gunther, 1939).
Fig. 61. *E. lanius* (Radford, 1946).
Fig. 62. *E. womersleyi* (Gunther, 1940).
Fig. 63. *E. brevipes* (Ewing, 1931).
Fig. 64. *Neoschöngastia namrui* (Wharton, 1946).
Fig. 65. *Euschöngastia lacunosa* (Gater, 1932).
Fig. 66. *Neoschöngastia monticola* (Wharton, 1946).
Fig. 67. *N. ewingi* (Wharton, 1946).
Fig. 68. *Euschöngastia nasuae* (Boshell & Kerr, 1942).
Fig. 69. *E. mutabilis* (Gater, 1932).
Fig. 70. *Neoschöngastia riversi* (Wharton, 1946).
Fig. 71. *Euschöngastia malayensis* (Gater, 1932).
Fig. 72. *E. mirafra* (Radford, 1942).
Fig. 73. *E. samboni* (Radford, 1942).
Fig. 74. *E. petrogale* (Womersley, 1934).
Fig. 75. *E. otomyia* (Radford, 1942).
Fig. 76. *E. cairnsensis* (Womersley, 1943).
Fig. 77. *E. foliata* (Gunther, 1940).
Fig. 78. *E. phascogale* (Womersley, 1943).
Fig. 79. *E. melomys* (Womersley, 1943).
Fig. 80. *E. perameles* (Womersley, 1939).
Fig. 81. *Neoschöngastia posekanyi* (Wharton, 1946).

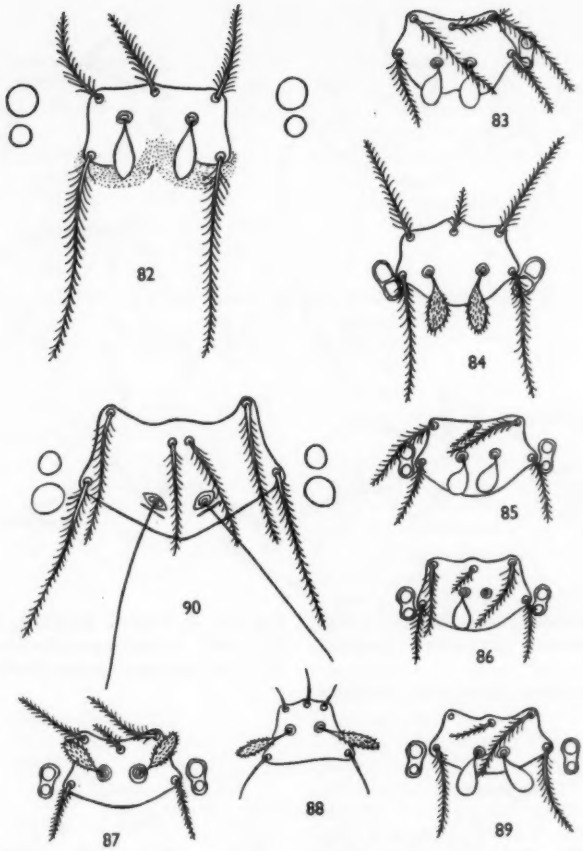


Fig. 82. *Neoschöngastia pauensis* Wharton, 1946.
Fig. 83. *Schöngastia taylori* Gunther, 1940.
Fig. 84. *Oenoschöngastia cana* Womersley, 1947.
Fig. 85. *Schöngastia willmanni* Radford, 1948.
Fig. 86. *S. maldiviensis* Radford, 1945.
Fig. 87. *S. vieta* Gater, 1932.
Fig. 88. *Doloisia manipurensis* (Radford, 1946).
Fig. 89. *Schöngastia andréi* Radford, 1948.
Fig. 90. *Hannemania ochotona* Radford, 1941.

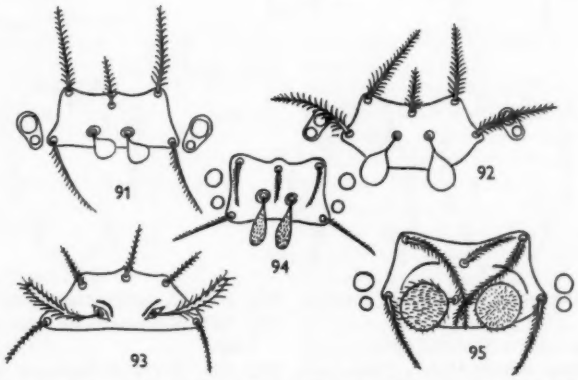


Fig. 91. *Euschöngastia hamiltoni* Brennan, 1947.
Fig. 92. *E. bushlandi* Philip, 1947.
Fig. 93. *E. yemenensis* Radford, 1952.
Fig. 94. *E. columbiae* (Boshell & Kerr, 1942).
Fig. 95. *Neoschöngastia kohlsi* Philip, 1946.

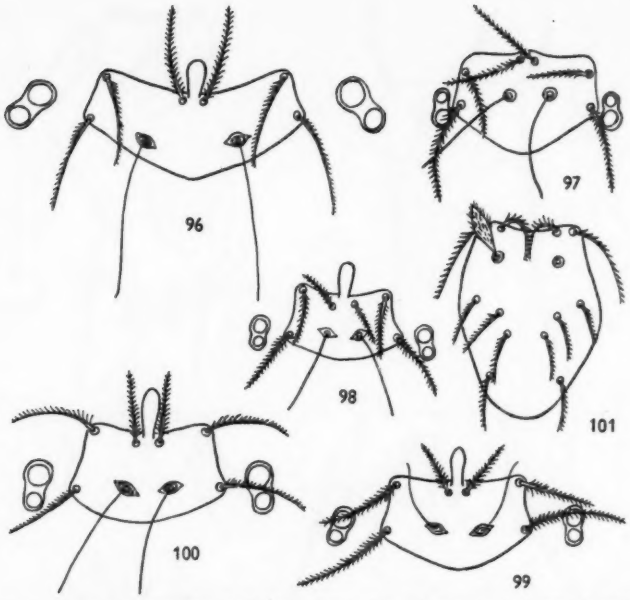


Fig. 96. *Acomatacarus gateri* (Radford, 1942).
Fig. 97. *Comatacarus atollensis* Radford, 1953.
Fig. 98. *Acomatacarus lawrencei* Radford, 1948.
Fig. 99. *A. keegani* Radford, 1953.
Fig. 100. *A. thallomyia* Radford, 1946.
Fig. 101. *Gateria hirsuta* Radford, 1946.

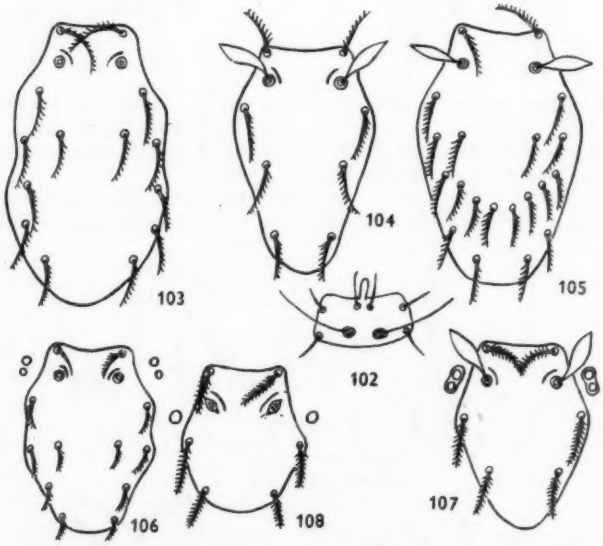


Fig. 102. *Acomatacarus arizonensis* Ewing, 1922.
Fig. 103. *Gateria ciliata* (Gater, 1932).
Fig. 104. *Gahrliepia cetrata* Gater, 1932.
Fig. 105. *Gateria fletcheri* (Gater, 1932).
Fig. 106. *G. rutila* (Gater, 1932).
Fig. 107. *Schöngastiella punctata* Radford, 1946.
Fig. 108. *S. brevis* Radford, 1946.

T. hamertoni Radford, 1942 (Fig. 7)	*Elaphe guttata* (L. 1766)
T. harrisoni Womersley, 1952	Bats
T. himizu Sasa *et al.* 1951	*Urotrichus talpoides hondonus* Thomas, 1908
	Microtus montebelloi Milne-Edwards, 1871
T. hoogstraali Radford, 1953	*Rattus r. rattus* L. 1758
T. incurva Womersley, 1952	Gecko
T. insolli Philip & Traub, 1950	*Eonycteris spelaea* (Dobson, 1871)
T. jacoti Womersley, 1952 n.nom. for *Trombiculoides scaber* Jacot, 1938, non *Trombidium scabrum* Say, 1821	—
T. jamesoni Brennan, 1948 (Fig. 45)	*Blarina b. brevicauda* Say, 1823
T. jonesae Brennan, 1952	*Peromyscus* sp.
T. jubbulporensis Womersley, 1952	*Rattus* sp.
T. kalrai Radford, 1952	*Rattus* sp.
T. kanzalwanensis Womersley, 1952	*Rattus* sp.
T. kashmiriensis Womersley, 1952	*Rattus* sp.
T. keiterti Fuller & Philip, 1950 (Fig. 11)	
T. kitasatoi Fukuzumi & Obata, 1951	—
	Apodemus s. speciosus Temminck, 1845
	Apodemus g. geisha Thomas, 1905
	Clethrionomys rufocanus smithii Thomas, 1905
	Microtus montebelloi Milne-Edwards, 1871
	Urotrichus talpoides hondonus Thomas, 1908
	Rattus r. rattus L. 1758
	Rattus n. norvegicus Berkenhout, 1769
T. knighti Radford, 1953	*Rhinolophus clivosus acrotis* Heuglin, 1861
T. kurdangensis Womersley, 1952	*Rattus* sp.
T. kuroshio Sasa & Kawashima, 1951	*Apodemus s. speciosus* Temminck, 1845
	Clethrionomys rufocanus smithii Thomas, 1905
T. lacertillae Boshell & Kerr, 1942	*Lacertillae* sp.
T. landazuri Boshell & Kerr, 1942	*Proechymis chrisaeolus* Thomas, 1898
T. legaci André, 1950	*Felis* sp.
	Taterillus e. emini Thomas, 1892
	Mylomys cunninghamei alberti Thomas, 1915
	Lemniscomys s. striatus L. 1758
	Gallus domesticus L. 1758
T. leveri Womersley, 1952	*Emballonura* sp.
T. lundbladi Womersley, 1952	*Scincus officinalis*
T. lygosomoides Womersley, 1952	*Lygosoma stanleyanum* Boulenger, 1897
T. macropus Womersley, 1936	*Macropus* sp.
T. mastomyia Radford, 1942 (Fig. 4)	*Mastomys coucha erythroleucus* Temminck, 1853
	—
T. mexicana Ewing, 1937	?
T. minor Berlese, 1905 (Fig. 6)	*Urotrichus talpoides hondonus* Thomas, 1908
T. miyairii Sasa *et al.* 1952	*Apodemus s. speciosus* Temminck, 1845
T. miyajimai Fukuzumi & Obata, 1951 (Fig. 15)	*Clethrionomys rufocanus smithii* Thomas, 1905
	Microtus montebelloi Milne-Edwards, 1871
T. miyazakii Sasa *et al.* 1951 (Fig. 16)	*Apodemus s. speciosus* Temminck, 1845
	Clethrionomys rufocanus smithii Thomas, 1905
T. montanensis Brennan, 1946 (Fig. 5)	*Cynomys l. ludovicianus* Ord, 1915
T. mounti Radford, 1953	*Nycteris d. damarensis* Peters, 1871
T. multisetosa Willmann, 1943	*Clethrionomys g. glareolus* Schreber, 1780
	Apodemus f. flavicollis Melchior, 1834
T. munda Gater, 1932 (Fig. 8)	*Rattus r. diardi* Jentink, 1879
T. muridia Womersley, 1952	*R. m. mulleri* Jentink, 1879
T. myops Vitzthum, 1931	*Molussus nasutus* Spix, 1823
T. myotis Ewing, 1929	*Myotis l. lucifugus* LeConte, 1831
T. natalensis Lawrence, 1949	*Rhinolophus geoffroyi zuluensis* Andersen, 1904
T. nativitatis Hoffmann, 1950	*Uta concinna* Dickerson, 1919

T. naultini Dumbleton, 1947	*Naultinus elegans* Gray, 1942
T. nigeriensis Ewing, 1928	*Funisciurus auriculatus oliviae* Dollman, 1911
T. nissani Dumbleton, 1947	?
T. nivara Lawrence, 1949	*Oedura nivaria* Boulenger, 1894
T. novae-hollandiae Hirst, 1929	*Rattus fuscipes greyi* Gray, 1841
T. orientalis Schluger, 1948 (Fig. 14)	*Clethrionomys rufocanus* Sundevall, 1846
T. parmifera Womersley, 1952 (Fig. 47)	*Mabuia multifasciata* (Kuhl, 1820)
T. pavlovskyi Schluger, 1948	*Apodemus agrarius agrarius* Pallas, 1778
	Microtus michnoi michnoi Kastschenko, 1910
	Rattus n. norvegicus Berkenhout, 1769
T. pearsoni Wharton, 1948	*Punomys lemminus* Osgood, 1943
T. pelta Womersley, 1952	*Rattus* sp.
T. philipi Womersley, 1952	*Hipposideros galeritus cervinus* Gould, 1853
T. piercei Ewing, 1933	*H. diadema griseus* Meyen, 1833
T. plumosa Radford, 1953	*Callosciurus p. pygerythrus* Geoffroy, 1832
T. pomeranzevi Schluger, 1948 (Fig. 13)	*Clethrionomys rufocanus* Sundevall, 1846
	Apodemus s. speciosus Temminck, 1845
T. potosina Hoffmann, 1950	*Neotoma macropus*
T. praomyia Radford, 1942 (Fig. 2)	*Praomys t. tullbergi* Thomas, 1894
T. psittaci Floch & Abbonenc, 1949	Parakeet
T. punctata Boshell & Kerr, 1942	*Agouti paca*
T. quadriense Womersley, 1943	Rats
T. radfordi Schluger, 1948	*Arvicola amphibius amphibius* L. 1758
T. rajoriensis Womersley, 1952	*Rattus* sp.
T. rhodesiana Lawrence, 1949	*Mabuya 5-taeniata margaritifer* Peters, 1854
T. robusta Gunther, 1941	*Pitta* sp.
T. russica Oudemans, 1910	Bats
T. samara Radford, 1953 (Fig. 48)	*Centropus melanops* Lesson, 1831
T. samboni Womersley, 1939 (Fig. 10)	—
T. sargenti Brennan, 1952	*Neotoma* sp.
T. schmitzi (Oudemans, 1914)	Bat
T. setosa Ewing, 1937	*Peromyscus g. gossypinus* LeConte, 1853
T. scottae Brennan, 1952	*P. t. truei* Shufeldt, 1886
T. shannoni Ewing, 1929	*Felis domesticus*
T. sobrina Womersley, 1952	*Lygosoma stanleyanum* Boulenger, 1897
T. southcotti Womersley, 1952	?
T. subakamushi Schulger, 1948	*Vespertilio superans* Thomas, 1899
T. sylvestris Audy & Traub, 1950	*Rattus s. sabanus* Thomas, 1887
T. tanaka-ryoi Kawashima & Sasa, 1952 (Fig. 49)	*Clethrionomys rufocanus smithii* Thomas, 1905
	Apodemus s. speciosus Temminck, 1845
T. taphozous Womersley, 1952	*Taphozous melanopogon* Temminck, 1841
T. tenjin Sasa *et al.* 1951	*Urotrichus talpoides hondonus* Thomas, 1908
T. teramurai Sasa *et al.* 1951	*Microtus montebelloi* Milne-Edwards, 1871
	Apodemus s. speciosus Temminck, 1845
	A. g. geisha Thomas, 1905
	Urotrichus talpoides hondonus Thomas, 1908
T. thori Womersley, 1952	*Leiolopisma melanopogon* Dumeril & Bibron, 1839
T. tithwalensis Womersley, 1952	*Rattus* sp.
T. tosa Sasa & Kawashima, 1951	*Rattus n. norvegicus* Berkenhout, 1769
	R. r. rattus L. 1758
T. vietzi Womersley, 1952	*Rattus* sp.
T. walchi Womersley, 1943	*R. r. diardi* Jentink, 1879
T. wichmanni (Oudemans, 1905)	*Goura coronata* (L. 1758)
syn. *T. mediocris* Berlese, 1912	
T. hirsti Gater, 1932	
T. hatori Womersley & Heaslip, 1943	
T. yasuokai Sasa *et al.* 1952	—
T. zachvatkini Schluger, 1948	?

Genus **Neotrombicula** Hirst, 1925

N. alaskensis Brennan & Wharton, 1950	*Hylocichla ustulata*
N. autumnalis (Shaw, 1790)	*Homo sapiens*
N. browni Brennan & Wharton, 1950	*Peromyscus m. maniculatus* Wagner, 1845
	Microtus longicaudus
	Tamiasciurus h. hudsonicus Erxleben, 1777
N. bisignata (Ewing, 1929)	*Microtus p. pennsylvanicus* Ord, 1815
N. californica (Ewing, 1942)	*Thomomys neglectus* Bailey, 1914
N. canestrinii (Buffa, 1899)	?
N. carterae Brennan & Wharton, 1950	*Peromyscus* sp.
N. cavicola (Ewing, 1931)	?
N. consueta Womersley, 1952	*Rattus b. bowersi* Andersen, 1879
N. dinehartae Brennan & Wharton, 1950 (Fig. 42)	*Peromyscus m. maniculatus* Wagner, 1845
N. eusignata Brennan & Wharton, 1950	*Tamias amoenus* Allen, 1890
	Citellus l. lateralis Say, 1823
N. farrelli Brennan & Wharton, 1950	*Peromyscus* sp.
N. goodpasteri Brennan & Wharton, 1950	*Pitemys pinetorum pinetorum* LeConte, 1829
	Microtus p. pennsylvanicus Ord, 1815
N. harperi (Ewing, 1928) (Fig. 32)	*Napaeozapus i. insignis* Miller, 1891
N. harrisoni Womersley, 1952	Bats
N. hastata (Gater, 1932)	*Rattus s. surifer* (Miller, 1900)
N. inopinatum (Oudemans, 1909)	?
N. jayewickremei Womersley, 1952	*Rattus r. kandianus* Kelaart, 1850
N. jewetti Brennan & Wharton, 1950 (Fig. 43)	*Microtus t. townsendi* Bachman, 1837
	Microtus longicaudus Merriam, 1888
	Clethrionomys californicus Merriam, 1890
	Peromyscus m. maniculatus Wagner, 1845
	Sorex t. trowbridgei Baird, 1857
	Peromyscus b. boylii Baird, 1855
N. kohlsi (Womersley, 1944)	*Homo sapiens* L.
N. lipovskyi Brennan & Wharton, 1950	*Neotoma* sp.
	Sylvilagus f. floridanus Allen, 1890
	Canis latrans Say, 1823
	Peromyscus sp.
	Reithrodontomys f. fulvescens Allen, 1894
	Neotoma f. florida Ord, 1818
N. microti (Ewing, 1928) (Fig. 34) syn. *Trombicula parkeri* Radford, 1942	*Microtus richardsoni macropus* Merriam, 1891
N. munda (Gater, 1932)	*Rattus r. diardi* Jentink, 1879
N. rara (Walch, 1923)	*Homo sapiens* L.
N. richmondi Brennan & Wharton, 1950	*Microtus p. pennsylvanicus* Ord, 1815
	Napaeozapus i. insignis Miller, 1891
	Ondatra zibethica L. 1758
	Clethrionomys g. gapperi Vigors, 1830
N. rohweri (Ewing, 1942)	*Troglodytes a. aedon* Vieillot, 1807
N. saperoi Radford, 1953	*Rattus r. rattus* L. 1758
N. sarcina Womersley, 1944	*Macropus cangaru* Muller, 1776
N. scincoides (Womersley, 1944)	*Lygosoma* (*Leiolopisma*) *bicarinatum* (McLeay 1877)
N. spicea (Gater, 1932) (Fig. 33)	*Rattus canus malaisia* Kloss, 1931
N. subsignata Brennan & Wharton, 1950	*Marmota f. flaviventris* Audubon & Bachman, 1841
	Citellus l. lateralis Say, 1823
	Sciurus motacilla
	Zapus h. hudsonius Zimmerman, 1780
	Microtus p. pennsylvanicus Ord, 1815
	Sylvilagus f. floridanus Allen, 1890

N. sylvilagi Brennan & Wharton, 1950 (Fig. 40)	*Sylvilagus f. floridanus* Allen, 1890
N. tovelli Womersley, 1952	*Synaptomys* sp.
N. traubi Womersley, 1952	Lizards
N. varians Brennan & Wharton, 1950	*Macacus a. assamensis* M'Clelland, 1839
N. waynensis Brennan & Wharton, 1950	*Citellus columbianus columbianus* Ord, 1815
N. whartoni Ewing, 1929 (Fig. 31)	*Melospiza georgiana* (Latham, 1790)
	Bird

Genus **Leptotrombidium** Nagayo *et al.* 1917

L. akamushi (Brumpt, 1910)	*Homo sapiens* L.
L. bhimtalensis Womersley, 1952	*Suncus* sp.
L. burmensis (Ewing, 1945)	*Rattus* sp.
L. deliensis (Walch, 1923)	*Homo sapiens* L.
	Rattus sp.
L. dux Womersley, 1952	*Rattus* sp.
L. fuji Kuwato *et al.* 1950 (Fig. 29)	*Apodemus s. speciosus* Temminck, 1845
L. fulleri (Ewing, 1945)	Soricidae
L. intermedia Nagayo *et al.* 1920	*Microtus montebelloi* Milne-Edwards, 1871
	Clethrionomys rufocanus smithii Thomas, 1905
	Apodemus s. speciosus Temminck, 1845
	A. g. geisha Thomas, 1905
	Rattus n. norvegicus Berkenhout, 1769
L. keukenshrijveri (Walch, 1923)	*Homo sapiens* L.
L. lanceolata Lawrence (in Wom. 1952)	*Bandicota bengalensis bengalensis* Gray & Hardwicke, 1833
	Rattus n. norvegicus Berkenhout, 1769
L. longiseta Womersley, 1952	?
L. macacus Womersley, 1952	*Macacus a. assamensis* M'Clelland, 1839
L. myzantha Womersley, 1952	—
L. pallida (Nagayo *et al.* 1919)	*Microtus montebelloi* Milne-Edwards, 1871
syn. *Trombicula burnsi* Sasa *et al.* 1950	*Apodemus s. speciosus* Temminck, 1845
T. murotoensis Sasa & Kawashima, 1951	*Clethrionomys rufocanus smithii* Thomas, 1905
	Mus mus molossinus Temminck, 1843
	Rattus r. rattus L. 1758
	R. n. norvegicus Berkenhout, 1769
	Apodemus g. geisha Thomas, 1905
	Urotrichus talpoides hondonus Thomas, 1908
L. palpalis (Nagayo *et al.* 1919)	*Microtus montebelloi* Milne-Edwards, 1871
	Apodemus s. speciosus Temminck, 1845
	Clethrionomys rufocanus smithii Thomas, 1905
	Rattus n. norvegicus Berkenhout, 1769
	Urotrichus talpoides hondonus Thomas, 1908
	Passer montanus saturatus
L. parapalpalis Womersley, 1952	*Rattus* sp.
L. puta Womersley, 1942	*Rattus* sp.
L. robusta (Gunther, 1941)	*Pitta mackloti* Temminck, 1834
L. scutellaris (Nagayo *et al.* 1920)	*Microtus montebelloi* Milne-Edwards, 1871
	Rattus n. norvegicus Berkenhout, 1769
	R. r. rattus L. 1758
	Apodemus s. speciosus Temminck, 1845
	A. g. geisha Thomas, 1905
	Urotrichus talpoides hondonus Thomas, 1905
	Canis sp.
	Felis sp.
L. villosa Womersley, 1952	*Rattus* sp.

Genus **Eutrombicula** Ewing, 1938

E. alfreddugesi (Oudemans, 1910) (Fig. 24)	*Homo sapiens* L.
syn. *Leptus irritans* Riley, 1873	
Tetranychus tlalsahuate Murray, 1877	
Trombicula cinnabaris Ewing, 1921	
T. vanommereni Shierbeek, 1937	
Leptus rileyi Oudemans, 1929	
E. agame Lawrence, 1949	*Agama hispida armata* Peters, 1854
E. anous Wharton, 1945 (Fig. 20)	*Anous stolidus* (L. 1758)
	Heteroscelus incanus (Gmelin, 1789)
E. batatus (L. 1758) (Fig. 23)	*Passalus* sp.
syn. *T. flui* van Thiel, 1930	
T. brasiliensis Ewing, 1925	
T. pastorae Boshell & Kerr, 1942	
E. bruyanti (Oudemans, 1910)	*Metachirus opossum* L. 1758
E. butantanensis (Fonseca, 1932)	*Homo sapiens* L.
E. coarctata (Berlese, 1888)	?
E. defecta Ewing, 1946	Spiny rat ?
E. draconensis Lawrence, 1949	*Pseudocordylus subviridis* Smith, 1838
E. dunni (Ewing, 1931)	*Dasyprocta punctata nuchalis* Goldman, 1917
E. gerrhosauri Lawrence, 1949	*Gerrhosaurus grandis* Boulenger, 1908
E. gliricolens (Hirst, 1915)	*Rattus r. rattus* L. 1758
E. gouldii (Oudemans, 1910) (Fig. 26)	*Dasyprocta a. agouti* L. 1766
syn. *E. helleri* Oudemans, 1911	
E. defecta Ewing, 1946	
E. gurneyi (Ewing, 1937)	*Eumeces fasciatus* L.
E. gymnodactyla Womersley & Kohls, 1947	*Gymnodactylus louisiadensis* De Vis, 1892
E. hirsti (Sambon, 1927) (Fig. 21)	*Homo sapiens* L.
E. hakei (Radford, 1946) (Fig. 27)	*Coluber radiatus* (Schlegel, 1837)
E. homopholis Lawrence, 1949	*Homopholis wahlbergii* Smith, 1849
E. insularis (Ewing, 1925)	*Anolis cybotes* Cope, 1862
E. isshikii (Sugimoto, 1938)	*Capella hardwickii* Gray, 1831
E. japa (Ribeyro & Bambaren, 1922)	?
E. lahillei Sig Thor & Willmann, 1947	*Homo sapiens* L.
syn. *Microtrombidium brumpti* Lahille, 1927	
E. manriquei Ewing, 1937	Humus
E. minutissima (Oudemans, 1910)	*Hipposideros cafer* Sundevall, 1846
E. montensis Lawrence, 1949	*Pseudocordylus subviridis* Smith, 1838
E. multisetosa (Ewing, 1943)	*Procyon* sp.
E. oenigma Lawrence, 1949	*Mabuya varia* Peters, 1867
E. ophidica (Fonseca, 1932)	*Ophis merremii* Wagler, 1824
E. pachydactyli Lawrence, 1949	*Pachydactylus l. laevigatus* Fischer, 1888
E. panamensis (Ewing, 1925)	*Sigmodon hispidus chiriquensis* Allen, 1904
E. pluvius (Wharton, 1945) (Fig. 25)	*Pluvius d. dominica* Muller, 1776
	Anous t. tenuirostris Temminck, 1823
	A. stolidus (L. 1758)
	Heteroscelus incanus (Gmelin, 1789)
E. rhodesiensis Lawrence, 1949	*Mabuya 5-taeniata margaritifer* (Peters, 1854)
E. rhoptropi Lawrence, 1949	*Rhoptropus afer* Peters, 1869
E. rioi (Gunther, 1939) (Fig. 19)	*Megapodius r. reinwardt* Dumont, 1823
E. splendens (Ewing, 1913) (Fig. 22)	?
syn. *A. masoni* Ewing, 1943	
E. sulae (Oudemans, 1910)	*Morus capensis* (Lichtenstein, 1823)
E. thomasi (Oudemans, 1910)	*Chraeomys j. jelskii* (Thomas, 1894)
E. tinami (Oudemans, 1910)	*Crypturus noctivagus* (Wied, 1820)
E. tropica (Ewing, 1925)	*Anaidia bitaeniata* Boulenger, 1884
E. vernalis Willmann, 1942	?
E. yorkei (Sambon, 1928)	*Hyla rubra* Daudin, 1803

Genus **Fonsecia** Radford, 1942

F. coluberina Radford, 1946	*Coluber radiatus* (Schlegel, 1837)
F. ewingi (Fonseca, 1932)	*Ophis merremii* Wagler, 1824
F. travassosi (Fonseca, 1935)	*Spilotes pullatus* L. 1758

Genus **Reidlinia** Oudemans, 1914

R. coeca Oudemans, 1914	*Rinolophus* sp.

Genus **Mackiena** Traub & Evans, 1950

M. empodiformia Traub & Evans, 1950	*Ploceus manyar peguensis* Stuart Baker, 1925

Genus **Heaslipia** Ewing, 1944

syn. *Trombiculoides* Womersley, 1943 pre. occ.

H. gateri (Womersley, 1943)	*Rattus r. argentiventer* Robinson & Kloss, 1916

Genus **Novotrombicula** Womersley & Kohls, 1947

N. owiensis Womersley & Kohls, 1947	?

Genus **Tragardhula** Berlese, 1912

syn. *Blankaartia* Berlese, 1912

Pentagonella Sig Thor, 1936

Megatrombicula Michener, 1946

T. acuscutellaris (Walch, 1922)	*Rattus* sp.
T. alleei (Ewing, 1926)	?
T. antropodis	?
T. ardeae (Tragardh, 1905)	*Ardea cinerea* L.
T. attenuata (Michener, 1946)	?
T. centropodis (Ewing, 1928)	*Centropus* sp.
T. desaleri (Methlagl, 1928)	*Homo sapiens* L.
T. fujigma (Philip & Fuller, 1950)	*Crocidura russula vorax* Allen, 1923
T. geckobia Womersley, 1952	Gecko
T. gymnodactyla (Womersley & Kohls, 1947)	*Gymnodactylus louisiadensis* De Vis, 1892
T. ilesi (Radford, 1948) (Fig. 36)	*Dendroaspis viridis* (Hallowell, 1844)
T. japonica (Tanaka, 1930)	*Homo sapiens* L.
T. mitamurai Sasa *et al.* 1950	*Apodemus s. speciosus* Temminck, 1845
	Microtus montebelloi Milne-Edwards, 1871
	Clethrionomys rufocanus smithii Thomas, 1905
	Urotrichus talpoides hondonus Thomas, 1908
T. muris (Oudemans, 1910)	*Apodemus s. sylvaticus* L. 1758
T. nagayoi Sasa *et al.* 1950	*Apodemus s. speciosus* Temminck, 1845
	Microtus montebelloi Milne-Edwards, 1871
	Clethrionomys rufocanus smithii Thomas, 1905
T. nilotica (Tragardh, 1905)	?
T. pentagona Womersley, 1952	On boots
T. peruviana (Ewing, 1926)	?
T. tamiyai (Philip & Fuller, 1950) (Fig. 30)	*Microtus montebelloi* Milne-Edwards, 1871
T. tragardhi (Oudemans, 1910)	*Cercopithecus a. aethiops* L. 1758
T. velascoi (Boshell & Kerr, 1942)	?

Genus **Trombiculindus** Radford, 1948

T. cuneatus Traub & Evans, 1951	*Crocidura* sp.
T. foliaceus Traub & Evans, 1951	*Crocidura* sp.
T. squamifera Womersley, 1952	Mouse
T. squamosus Radford, 1948	*Rattus* sp.

Genus **Crotiscus** Ewing, 1944

C. desdentata (Boshell & Kerr, 1942)	*Praechymis chrisaeolus* Thomas, 1898

Genus **Neoschöngastia** Ewing, 1929

syn. *Paraschöngastia* Womersley, 1939

N. americana (Hirst, 1921) (Fig. 56)	*Gallus domesticus* L. 1758
N. a. solomonis Wharton, 1946 (Fig. 52)	*Hirundo tahitica* Gmelin, 1788
N. atollensis Wharton, 1946 (Fig. 53)	*Numenius phaeopus* L. 1758
N. backhousei Gunther 1939	*Megapodius duperreyi*
N. bougainvillensis Wharton, 1946 (Fig. 51)	*Hirundo tahitica* Gmelin, 1788
N. carveri Wharton, 1946 (Fig. 50)	*Numenius phaeopus* L. 1758
	Demigretta sacra (Gmelin, 1788)
	Pluvialis d. dominica Muller, 1776
	Sterna d. dougalii Montagu, 1813
	Monticola solitarius (L. 1758)
	Heteroscelus incanus (Gmelin, 1789)
N. dalmati Brennan, 1951	*Momotus lessonii* Lesson, 1842
N. dubia Gunther, 1939	*Megapodius duperreyi*
N. egretta Wharton, 1946 (Fig. 54)	*Egretta intermedia* (Wagler, 1829)
	Demigretta sacra (Gmelin, 1788)
	Anous stolidus (L. 1758)
	Pluvialis d. dominica Muller, 1776
N. entomyza Womersley, 1952	*Entomyzon cyanotis* Latham, 1801
	Struthidia cinerea Gould, 1837
	Pomatostomus temporalis
N. ewingi Wharton, 1946 (Fig. 67)	*Egretta intermedia* (Wagler, 1829)
	Gallus gallus sp.
	Pluvialis d. dominica Muller, 1776
	Heteroscelus incanus (Gmelin, 1789)
N. fullbergae Brennan, 1948	*Odocoileus h. hemionus* Rafinesque, 1817
N. gallinarum (Hatori, 1920)	*Gallus g. domesticus* Briss,
N. ikaoensis Sasa *et al.* 1951	*Apodemus s. speciosus* Temminck, 1845
N. kohlsi Philip, 1946	*Rattus m. mindanensis* Mearns, 1905
N. mcqueeni Womersley, 1952	?
N. monticola Wharton, 1946 (Fig. 66)	*Monticola solitarius* (L. 1758)
N. namrui Wharton, 1946 (Fig. 64)	*Numenius phaeopus* L. 1758
	Gygis alba (Sparrman, 1786)
	Heteroscelus incanus (Gmelin, 1789)
	Pluvialis d. dominica Muller, 1776
N. novae-hollandia Wharton, 1946	*Rattus r. greyi* Gray, 1841
N. owiensis Womersley, 1952	On boots
N. paenitens Brennan, 1952	*Petrochelidon pyrrhonota pyrrhonota* (*Vieillot*, 1817)
syn. *N. kohlsi* Brennan, 1952 pre. occ.	
N. pauensis Wharton, 1946 (Fig. 82)	*Pluvialis d. dominica* Muller, 1776
N. posekanyi Wharton, 1946 (Fig. 81)	*Monticola solitarius* (L. 1758)
	Streptopelia orientalis Latham, 1790
N. retrocincta Gunther, 1939	*Megapodius duperreyi*

N. riversi Wharton, 1946 (Fig. 70)	*Eurystomus orientalis* (L. 1766)
	Haliacetus sanfordi Mayr, 1935
	Falco sererus Horsfield, 1822
	Halcyon chloris (Boddaert, 1783)
N. salmi (Oudemans, 1922)	?
N. strongi Wharton, 1946	*Numenius phaeopus* L. 1758
	Arenaria interpres L. 1758
N. struthidia Womersley, 1952	*Struthidia cinerea* Gould, 1837
N. thomasi (Radford, 1946) (Fig. 57)	*Lanius nasutus* Scopoli, 1786
N. yeomansi Gunther, 1939 (Fig. 60)	*Megapodius r. reinwardt* Dumont, 1823

Genus **Ascoschöngastia** Ewing, 1946

A. aethiopica (Hirst, 1926)	Small grey bat
A. capillata Radford, 1952	*Rattus* sp.
A. malayensis (Gater, 1932)	*Rattus canus malaisia* Kloss, 1931
A. masta Traub & Sundermeyer, 1950	*R. r. rattus* L. 1758
A. ophicola Lawrence, 1949	*Booedon lineatus* Dumeril & Bibron, 1854
A. viperina Lawrence, 1949	*Causus rhombeatus* Lichtenstein, 1823

Genus **Euschöngastia** Ewing, 1938

E. aethomyia (Radford, 1942) (Fig. 58)	*Thallomys namaquensis grahami* Roberts, 1915
E. africana (Radford, 1948)	*Otomys tugelensis pretoriae* Roberts, 1929
E. americana Ewing, 1938	*Eutamias* sp.
E. annulata Lawrence, 1949	*Elephantulus rupestris jamesoni* Chubb, 1909
E. antipodianum (Hirst, 1929)	*Rattus g. greyi* Gray, 1841
E. audyi (Womersley, 1952)	?
E. brevipalpis (André, 1946)	*Meriones l. libycus* Lichtenstein, 1823
E. brevipes (Ewing, 1931) (Fig. 63)	*Peromyscus leucopus noveboracensis* Fischer, 1829
E. brasiliensis (Fonseca, 1935)	*Rattus* sp.
E. bushlandi Philip, 1947 (Fig. 92)	*Megapodius* sp.
E. cairnsensis (Womersley, 1943) (Fig. 76)	*Rattus* sp.
E. c. var. *gateri* (Womersley, 1943)	*Rattus* sp.
E. californica (Ewing, 1925)	Ground squirrel
E. capensis Lawrence, 1949	*Eremias lineo-ocellata pulchella* Gray, 1845
E. cassiope Womersley, 1952	On boots
E. cercopitheci (Tragardh, 1904)	*Cercopithecus a. aethiops* L. 1758
E. cockingsi (Radford, 1946)	*Rattus rattus rufescens* Gray, 1837
E. columbiae (Boshell & Kerr, 1942) (Fig. 94)	*Proechymis chrisaeolus* Thomas, 1898
E. comata Womersley, 1952	?
E. coorongense (Hirst, 1929)	*Rattus l. lutreolus* Gray, 1841
E. cordiremus Brennan, 1948	*Peromyscus maniculatus artemisiae* Rhoads, 1894
E. costulata Willmann, 1952	*Apodemus s. sylvaticus* L. 1758
	A. f. flavicollis Melchior, 1834
	Clethrionomys g. glareolus (Schreber, 1780)
E. covelli (Radford, 1952)	*Callosciurus p. pygerythrus* Geoffroy, 1832
E. criceticola Brennan, 1948	*Peromyscus maniculatus artemisiae* Rhoads, 1894
E. crinita Womersley, 1952	*Echymipera k. kalubu* Lesson, 1828
E. crocidurae Lawrence, 1949	*Crocidura flavescens* Geoffroy, 1827
E. dasycerci (Hirst, 1929)	*Dasycercus cristicauda* Krefft, 1867
E. dasyproctae (Ewing, 1937)	*Dasyprocta v. variegata* Tschudi, 1845
E. debilis (Gater, 1932)	*Rattus c. cremoriventer* Miller, 1900
E. derricki (Womersley, 1939)	*R. l. lutreolus* Gray, 1841
E. dumosa Womersley, 1952	On boots

E. dutoiti (Radford, 1948)	*Saccostomus c. campestris* Peters, 1846
E. echymipera (Womersley & Kohls, 1947)	*Echymipera cockerelli* Ramsay, 1877
E. edwardsi (Gunther, 1937)	Bush fowl
E. foliata (Gunther, 1940) (Fig. 77)	Wallaby
E. gerrhosauri Lawrence, 1949	*Gerrhosaurus flavigularis* Wiegman, 1828
E. globulare (Walch, 1923)	Rats
E. guntheri (Radford, 1942) (Fig. 55)	*Ochotona* sp.
E. hamiltoni Brennan, 1947 (Fig. 91)	*Eptesicus f. fuscus* (Beauvois, 1796)
E. hastata (Gater, 1932)	*Rattus s. surifer* Miller, 1900
E. heaslipi (Womersley, 1943)	*Rattus* sp.
E. hirsti (Womersley, 1943)	*Melomys c. cervinipes* Gould, 1852
E. hirsuta (Boshell & Kerr, 1942)	*Proechymis chrisaeolus* Thomas, 1898
E. indica (Hirst, 1915)	*Bandicota b. bengalensis* Gray & Hardwicke, 1833
E. innisfailensis (Womersley, 1943)	*Melomys cervinipes littoralis* Lönneberg, 1916
E. kalaharica Lawrence, 1949	*Ichnotropis squamulosa* Peters, 1854
E. kohlsi (Ewing, 1946) (Fig. 95)	*Rattus m. mindanensis* Mearns, 1905
E. labuanensis (Womersley, 1952)	*Rattus* sp.
E. lacerta Brennan, 1948	*Sceloporus o. occidentalis* Baird & Girard, 1852
E. lacunosa (Gater, 1932) (Fig. 65)	*Rattus sabanus vociferans* Miller, 1900
E. lanius (Radford, 1946) (Fig. 61)	*Lanius nasutus* Scopoli, 1786
E. lappacea (Womersley, 1952)	*Rattus* sp.
E. lawrencei (Womersley, 1952) syn. *N. guntheri* Womersley, 1943 pre. occ.	Rats
E. lipoxena Womersley, 1952	Bats
E. longispina (Radford, 1948) (Fig. 59)	*Thallomys namaquensis grahami* Roberts, 1915
E. lorius (Gunther, 1939)	*Lorius voratus* spp.
E. luteodema Brennan, 1948	*Marmota flaviventer nosophora* Howell, 1914
E. mackerrasae (Womersley, 1952)	Free living
E. matoppoanus Lawrence, 1949	*Platysaurus guttatus rhodesiensis* Fitzsimons, 1941
E. malayensis (Gater, 1932) (Fig. 71)	*Rattus canus malaisia* Kloss, 1931
E. masta (Traub & Sundermeyer, 1950)	*R. r. rattus* L. 1758
	Tupaiaglis belangeri Wagner, 1841
E. mccullochi (Womersley, 1944)	On boots
E. melomys (Womersley, 1943) (Fig. 79)	*Melomys cervinipes littoralis* Lönneberg, 1916
E. mirafra (Radford, 1942) (Fig. 72)	*Mirafra africana tropicalis* Hartert, 1900
E. miyagawai Sasa *et al.* 1951	*Apodemus s. speciosus* Temminck, 1845
	Emberiza variabilis
	Parus varius owstoni
	Turdus celaenops celaenops
	Bambusicola thoracica thoracica
	Phasianus versicolor
E. mohri (Womersley, 1952)	*Echymipera k. kalubu* Lesson, 1828
E. muris (Walch, 1923)	*Rattus* sp.
E. mutabilis (Gater, 1932) (Fig. 69)	*R. sabanus vociferans* Miller, 1900
E. nadchatrami (Womersley, 1952)	*R. s. sabanus* Thomas, 1887
E. nasuae (Boshell & Kerr, 1942) (Fig. 68)	*Nasua candace* Thomas, 1912
E. nausheraensis (Womersley, 1952)	Mouse
E. nunezi (Hoffmann, 1944)	*Homo sapiens* L.
E. origensis Lawrence, 1949	*Tropidasaura essexi* Hewitt, 1927
E. otomyia (Radford, 1942) (Fig. 75)	*Otomys tugelensis pretoriae* Roberts, 1929
E. perameles (Womersley, 1939) (Fig. 80)	*Isoodon torosus* Ramsay, 1877
E. peregrina (Womersley, 1952)	On boots
E. peromysci Ewing, 1929	*Peromyscus leucopus noveboracensis* Fischer, 1829
E. petrogale (Womersley, 1934)	Wallaby

E. phascogale (Womersley, 1943) (Fig. 74)	*Phascogale* sp.
E. philippensis (Ewing, 1946) (Fig. 78)	*Rattus m. mindanensis* Mearns, 1905
E. phylloti Wharton, 1948	*Phyllotis d. darwinii* Waterhouse, 1837
	Chinchillula sahamae Thomas, 1898
E. pipistrelli Brennan, 1947	*Pipistrellus s. subflavus* Curier, 1832
syn. *E. miricoxa* Brennan, 1948	
E. pseudomys (Womersley, 1952)	*Pseudomys* sp.
E. queenslandica (Womersley, 1939)	*Rattus fuscipes assimilis* Gould, 1858
E. rattus (Womersley, 1943)	*R. fuscipes assimilis* Gould, 1858
E. raui (Womersley, 1952)	*Leiolopisma guichenoti* Dumeril & Bibron,
E. rectangulare (Womersley, 1952)	?
E. rhodesiensis (Lawrence, 1949)	*Mabuya 5-taeniata margaritifer* (Peters, 1854)
E. samboni (Radford, 1942) (Fig. 73)	Pika
E. sarawakensis (Womersley, 1952)	*Sorex minutus gymnurus* Chaworth-Musters, 1932
E. scelopori (Ewing, 1931)	*Sceloporus spinosus* Wiegman, 1828
E. sciuricola (Ewing, 1925)	*Tamiasciurus hundsonicus richardsoni* Buchman, 1838
E. shieldsi (Gunther, 1941)	*Melomys moncktoni rubex* Thomas, 1922
E. signata (Womersley, 1952)	On boots
E. signator (Ewing, 1931)	*Neotoma* sp.
E. similis (Womersley, 1943)	*Rattus* sp.
E. smithi (Womersley, 1939)	*R. fuscipes assimilis* Gould, 1858
E. soekaboemiensis (Takekawa, 1945)	*R. r. diardi* Jentink, 1879
E. transvaalensis Lawrence, 1949	*Ichnotropis squamulosa* Peters, 1854
E. traubi (Womersley, 1952)	*Suncus* sp.
E. trichosuri (Womersley, 1939)	*Trichosurus vulpecula* Kerr, 1792
E. tropidusauri (Lawrence, 1949)	*Tropidosaura essexi* Hewitt, 1927
E. trouessarti (Oudemans, 1910)	*Metachirus opossum* L. 1758
E. uromys (Womersley & Kohls, 1947)	*Uromys caudimaculatus multiplicatus* Jentink, 1907
E. westralensis (Womersley, 1943)	*Felis domesticus* L.
E. womersleyi (Gunther, 1940) (Fig. 62)	Wallaby
E. wongabelensis (Womersley, 1952)	Water rat ?
E. xerothermobia (Willmann, 1942)	?
E. yemenensis (Radford, 1953) (Fig. 93)	*Meriones rex* Thomas, 1902

Genus **Schöngastia** Oudemans, 1910

S. algerica (André, 1932)	?
S. andréi Radford, 1948 (Fig. 89)	*Lophuromys sikapusi ansorgei* de Winton, 1896
S. bidentata Womersley, 1952	Skink
S. blestowei Gunther, 1939	*Megapodius r. reinwardt* Dumont, 1823
S. b. var. *megapodius* Womersley, 1943	Bush fowl
S. berlesei (Oudemans, 1903)	*Riparia r. riparia* (L. 1758)
S. gerrhosauri Lawrence, 1949	*Gerrhosaurus flavigularis* Wiegmann, 1828
S. guyanensis Floch & Abonnenc, 1941	*Homo sapiens* L.
S. impar (Gunther, 1939)	*Echymipera cockerelli* Ramsay, 1877
	Peroryctes raffrayana
	Melomys moncktoni
	M. stalkeri
	M. rubex Thomas, 1922
	Rattus ringens
S. jamesi Gunther, 1939	*Megapodius r. reinwardt* Dumont, 1823
	Echymipera cockerelli Ramsay, 1877
S. katonis Womersley, 1943	?
S. lewthwaitei Womersley, 1952	*Tupaia glis belangeri* Wanger, 1841
S. lynni Ewing, 1942	Frog

S. mabuyana Lawrence, 1949	*Mabuya striata* Peters, 1844
S. madecassa André, 1949	Chameleon
S. maldiviensis Radford, 1945 (Fig. 86)	*Calotes versicolor* Dumeril & Bibron, 1841
S. monticola Lawrence, 1949	*Pseudocordylus subviridis* Smith, 1838
S. oculicola Womersley, 1952	*Leggada booduga* Gray, 1837
S. oudemansi Walch, 1922	*Rattus* sp.
S. philipi Womersley & Kohls, 1947 (Fig. 35)	*Leiolopisma albertisi* (Peters & Doria, 1878)
S. platisauri Lawrence, 1949	*Platysaurus guttatus rhodesiensis* Fitzsimons, 1941
S. pseudocordyli Lawrence, 1949	*Pseudocordylus spinosus* Fitzsimons, 1947
S. pseudoschuffneri (Walch, 1927)	*Homo sapiens* L.
S. pusilla Womersley, 1944	?
S. samoaensis Womersley, 1952	?
S. schuffneri (Walch, 1922)	*H. sapiens* L.
S. scincicola Lawrence, 1949	*Mabuya varia* Peters, 1867
S. taylori Gunther, 1940 (Fig. 83)	Wallaby
S. vandersandei (Oudemans, 1905)	*Homo sapiens* L.
S. vieta Gater, 1932 (Fig. 87)	*Rattus r. diardi* Jentink, 1879
S. westraliensis (Womersley, 1934)	*Felis domesticus*
S. whartoni Womersley, 1952	*Homo sapiens* L.
S. willmanni Radford, 1948 (Fig. 85)	*Arvicanthis abyssinicus rubescens* Wroughton, 1909

Genus **Radfordiana** Womersley, 1952

R. rostrata Womersley, 1952	?

Genus **Oenoschöngastia** Womersley & Kohls, 1947

O. cana Womersley & Kohls, 1947 (Fig. 84)	Mound of brush turkey

Genus **Tecomatlana** Hoffmann, 1947

T. sundovali Hoffmann, 1947	Bat

Genus **Trisetica** Traub & Evans, 1950

T. melvini Traub & Evans, 1950	Bat

Genus **Myotrombicula** Womersley, 1943

M. vespertilionis Womersley, 1943	Bats

Genus **Guntherana** Womersley, 1943

syn. *Guntheria* Womersley, 1936 pre. occ.

G. bipygalis (Gunther, 1939)	Rats
syn. *Neoschöngastia callipygos* Gunther, 1939	*Isoodon torosus* Ramsay, 1877
G. parana Womersley, 1944	*Homo sapiens* L.

Genus **Doloisia** Oudemans, 1910

D. manipurensis (Radford, 1946) (Fig. 88)	*Rattus rattus rufescens* Gray, 1837
D. okabei Sasa *et al.* 1952	*Urotrichus talpoides hondonus* Thomas, 1908
D. synoti Oudemans, 1910	*Barbastella barbastella* Shreber, 1774

Genus **Sauriscus** Lawrence, 1949

S. ewingi Lawrence, 1949	*Pachydactylus bibroni turneri* Gray, 1864

Genus **Endotrombicula** Ewing, 1931

E. bufonica Radford, 1953	*Bufo orientalis* Werner,
E. madagascariensis (Sambon, 1928)	*Mantidactylus luteus* Metheun & Hewitt, 1913
E. penetrans Ewing, 1931	*Arthroleptis minutus* Boulenger, 1884
E. pillersi (Sambon, 1928)	*Phrynobatrachus natalensis* (Smith, 1849)

Genus **Phrynacarus** Lawrence, 1949

P. fitzsimonsi Lawrence, 1949	*Heleophryne regis* Hewitt, 1909

Family LEEUWENHOEKIDAE Womersley, 1945

Subfamily LEEUWENHOEKIINAE Womersley, 1944

Genus **Leeuwenhoekia** Oudemans, 1911

L. verduni (Oudemans, 1910)	*Metachirus opossum* L. 1758

Genus **Odontacarus** Ewing, 1929

O. australis (Ewing, 1929)	*Tropiduras peruvianus* Lesson, 1830
O. dentatus (Ewing, 1925)	*Odocoileus couesi* Coues & Yarrow, 1875
O. gymnodactyli (Ewing, 1925)	*Gymnodactylus lawderanus* Stoliczka, 1872

Genus **Hannemania** Oudemans, 1911

H. argentina Lahille, 1927	*Pleurodema bibroni* Tschudi, 1838
H. dunni Sambon, 1928	*Desmognathus f. fuscus* (Rafinesque, 1820)
H. edwardsi Sambon, 1928	*Bufo variegatus* Gunther, 1873
H. eltoni Sambon, 1928	*Rana sphenocephala* (Cope, 1886)
H. hepatica Fonseca, 1935	*Leptodactylus ocellatus* L. 1758
H. hobdayi Sambon, 1928	*Pleurodema bufonina* Bell, 1843
H. hylae Ewing, 1925	*Hyla arenicolor* Cope, 1866
H. hylodeus (Oudemans, 1910)	*Hylodes* sp.
H. newsteadi Sambon, 1928	*Hyla rubra* Daudin, 1803
H. ochotona Radford, 1942 (Fig. 90)	*Ochotona* sp.
H. pattoni Sambon, 1928	*Eupsophus taeniatus* Girard, 1854
H. penetrans Ewing, 1931	*Rana pipiens* Schreber, 1782
H. rouxi Oudemans, 1917	*Hyla aurea* Lesson, 1830
H. samboni Ewing, 1931	*Pleurodema bibroni* Tschudi, 1838
H. stephensi Sambon, 1928	*Eleuthrodactylus gollmeri* (Peters, 1863)
H. vellae Dumbleton, 1947	Flying fox

Genus **Brennanella** Radford, 1953

B. longispina Radford, 1953	*Rhinolophus clivosus acrotis* Heuglin, 1861

Genus **Acomatacarus** Ewing, 1942

A. adelaideae (Womersley, 1944)	*Felis domesticus*
A. arabicus Radford, 1953	*Stenodactylus pulcher* Andersen,
A. arizonensis Ewing, 1942 (Fig. 102)	*Callisciurus draconoides ventralis*
A. australiensis (Hirst, 1925)	*Homo sapiens*
A. athertonensis (Womersley, 1945)	?

A. audyi Radford, 1946	Bird (babbler?)
A. barrinensis (Womersley, 1945)	?
A. bakeri Hoffmann, 1951	*Peromyscus truei gratus* (Merriam, 1898)
A. chiapanensis Hoffmann, 1948	Badger
A. echidnus (Womersley, 1945)	Echidna
A. galli Ewing, 1946	*Gallus* sp.
A. gateri (Radford, 1942) (Fig. 96)	*Tatera b. brantsi* Smith, 1836
A. geckobius Lawrence, 1949	*Pachydactylus bicolor* Hewitt, 1926
A. hirsti (Womersley, 1944)	*Homo sapiens* L.
A. hirsuta (Ewing, 1931)	*Perognathus* sp.
A. jaegerskioeldi (Oudemans, 1911)	Under stones
A. keegani Radford, 1953 (Fig. 99)	*Centropus melanops* Lesson, 1831
A. lacertae Lawrence, 1949	*Eremias lineo-ocellata* Gray, 1845
A. lawrencei Radford, 1948 (Fig. 98)	*Thallomys namaquensis grahami* Roberts, 1915
A. longipes (Womersley, 1945)	*Podargus* sp.
A. lygosomae Dumbleton, 1947	*Lygosoma grande* Gray, 1845
A. mabuyana Lawrence, 1949	*Mabuya varia* Peters, 1867
A. mccullochi (Womersley, 1944)	*Homo sapiens* L.
A. namaquensis Lawrence, 1949	*Gerrhosaurus typicus* Gray, 1845
A. nova-guinea (Womersley, 1944)	*Gymnorhina* sp.
A. paradoxa (André, 1943)	*Buthus gibbosus*
A. polydiscum (Oudemans, 1910)	*Hipposideros cafer* Sundevall, 1846
A. southcotti (Womersley, 1944)	*Lygosoma* sp.
A. tenuitarsus Lawrence, 1949	*Mabuya varia* Peters, 1867
A. thallomyia Radford, 1946 (Fig. 100)	*Thallomys namaquensis grahami* Roberts, 1915
A. theileri Radford, 1946	*Saccostomus c. campestris* Peters, 1846
A. tuberculatis Brennan, 1952	*Heteromys a. anomalus* Thompson, 1815

Genus **Comatacarus** Ewing, 1942

C. americanus Ewing, 1942	Western mole
C. atollensis Radford, 1953 (Fig. 97)	*Rattus n. norvegicus* Berkenhout, 1769
C. occidentalis Ewing, 1942	Common ground squirrel

Genus **Xenacarus** Greenberg, 1951

X. plumosus Greenberg, 1951	*Peromyscus m. maniculatus* Wagner, 1845

Genus **Chatia** Brennan, 1946

C. setosa Brennan, 1946	*Tamiasciurus hudsonicus richardsoni* Bachman, 1838

Genus **Austracarus** Lawrence, 1949

A. procaviae Lawrence, 1949	*Procavia c. capensis* Pallas, 1766

Genus **Hyracarus** Lawrence, 1949

H. longipilosus Lawrence, 1949	*Procavia c. capensis* Pallas, 1766
H. natalensis Lawrence, 1949	*Mabuya striata* Peters, 1844
H. typicus Lawrence, 1949	*Procavia c. capensis* Pallas, 1766

Genus **Austrombicula** Lawrence, 1949

A. womersleyi (Lawrence, 1948)	*Amblysomus hottentotus longiceps* (Broom, 1907)

Genus **Womersleyia** Radford, 1945

W. minuta Radford, 1945	Grasshoppers

Genus **Whartonia** Ewing, 1944

W. nudosetosa (Wharton, 1938)	*Artibeus jamaicensis yucatanicus* Allen, 1904
W. perplexa (Brennan, 1947)	*Eptesicus fuscus pallidus* Young, 1908
W. whartoni Hoffmann, 1951	*Baiomys m. musculus* Merriam, 1892

Subfamily WALCHIINAE Ewing, 1946

Genus **Walchia** Ewing, 1931

W. americana Ewing, 1942	Cotton mouse
W. brennani Womersley, 1952	*Rhinosciurus l. laticaudatus* Müller & Schlegel, 1839
W. disparunguis (Oudemans, 1929)	*Rattus rattus* ssp.
W. enode Gater, 1932	*R. mulleri validus* Miller, 1900
W. glabrum (Walch, 1927)	*Rattus* sp.
W. lewthwaitei Gater, 1932	*R. rattus (diardi ?)*
W. morobensis Gunther, 1939	*R. exulans browni* Alston, 1877
W. rustica Gater, 1932	*R. s. surifer* Miller, 1900
W. turmalis Gater, 1932	*R. sabanus vociferans* Miller, 1900

Genus **Anomalaspis** Brennan, 1952

A. ambiguus Brennan, 1952	*Heteromys a. anomalus* Thompson, 1815

Genus **Gahrliepia** Oudemans, 1912

G. cetrata Gater, 1932 (Fig. 104)	*Rattus edwardsi ciliatus* Bonhote, 1900
G. decora Womersley, 1952	*R. s. sabanus* Thomas, 1887
G. homunguis Abdussalam, 1939	*R. r. rattus* L. 1758
G. insigne Womersley, 1952	*Tupaia glis glis*
G. ornata Womersley, 1952	*Rattus s. sabanus* Thomas, 1887
G. saduski Womersley, 1952	*Mus sp.*

Genus **Gateria** Ewing, 1938

G. ciliata (Gater, 1932) (Fig. 103)	*Rattus v. validus* Miller, 1900
G. crocidura Radford, 1946	*Suncus (Crocidura) murinus fulvocinereus* (Andersen, 1877)
G. fletcheri (Gater, 1932) (Fig. 105)	*Rattus r. diardi* Jentink, 1879
G. hirsuta Radford, 1946 (Fig. 101)	*Suncus (Crocidura) murinus fulvocinereus* (Andersen, 1877)
G. lancearia Radford, 1946	*Talpa m. micrura* Hodgson, 1841
G. longipili Radford, 1946	*Suncus (Crocidura) murinus fulvocinereus* (Andersen, 1877)
G. romeri Womersley, 1952	*Rattus r. rattus* L. 1758
G. rutila (Gater, 1932) (Fig. 106)	*R. v. validus* Miller, 1900
G. spinulosa Radford, 1946	*Suncus (Crocidura) murinus fulvocinereus* (Andersen, 1877)

Genus **Pseudoschöngastia** Lipovsky, 1951

P. diazi (Hoffmann, 1948)	Badger
P. farneri Lipovsky, 1951	*Cryptotis parva* Say, 1823
	Peromyscus maniculatus Wagner, 1845
P. guatemalensis Brennan, 1952	*Sylvilagus floridanus chiapensis* Nelson, 1904

P. hungerfordi Lipovsky, 1951	*Sylvilagus f. floridanus* Allen, 1890
	Peromyscus maniculatus Wagner, 1845
	Neotoma m. micropus Baird, 1855
	N. floridana attwateri Nearns, 1897
	Reithrodontomys f. fulvescens Allen, 1894
P. occidentalis Brennan, 1952	*Peromyscus b. boylii* Baird, 1855
	P. c. californicus Gambel, 1848

Genus **Schöngastiella** Hirst, 1915

S. bengalensis Hirst, 1915	*Rattus r. rattus* L. 1758
S. brevis Radford, 1946 (Fig. 108)	*R. rattus rufescens* Gray, 1837
S. ceylonica Womersley, 1952	*R. r. kandianus* Kelaart, 1850
S. kumaonensis Womersley, 1952	*Suncus* sp.
S. ligula Radford, 1946	*Rattus rattus rufescens* Gray, 1837
S. punctata Radford, 1946 (Fig. 107)	*Suncus* (*Crocidura*) *murinus fulvocinereus* (Andersen, 1877)

Subfamily Apoloniinae Wharton, 1947

Genus **Apolonia** Torres & Braga, 1939

A. tigipioensis Torres & Braga, 1939	*Gallus domesticus* L. 1758

Genus **Womersia** Wharton, 1947

W. strandtmani Wharton, 1947	*Pelecanus occidentalis* L. 1766

Genus **Sauracarella** Lawrence, 1949

S. africana Lawrence, 1949	*Tetradactylus s. seps* (L. 1758)
S. montanus Lawrence, 1949	*Tropidosaura cottrelli* (Hewitt, 1925)
S. whartoni Lawrence, 1949	*Pseudocordylus subviridis* Smith, 1838

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RHIPICEPHALUS HURTI N.SP. (IXODIDAE) FROM KENYA GAME AND DOMESTIC ANIMALS

By S. G. WILSON

Chief Field Zoologist, Veterinary Department, Kenya*

(With 6 Figures in the Text)

MALE (Figs. 1–4)

Salient features. A medium-sized, inornate, dark-brown tick with broad basis capituli, auriculae anterior and acute. Conscutum with numerous, shallow, medium-sized, closely spaced punctations. The punctations are usually discrete, but a few may coalesce; in places they tend to linear arrangement; they are denser in the region of the posterior dorsal furrows than anteriorly, and they tend to be finer at the sides. Cervical pits short, deeply defined. Lateral grooves shallow, with five or six large punctations. Anal plates elongate and triangular, with a slight protuberance on the internal baso-lateral angle, their internal borders slightly concave, their posterior borders rounded. Coxae I just visible dorsally.

Detailed description

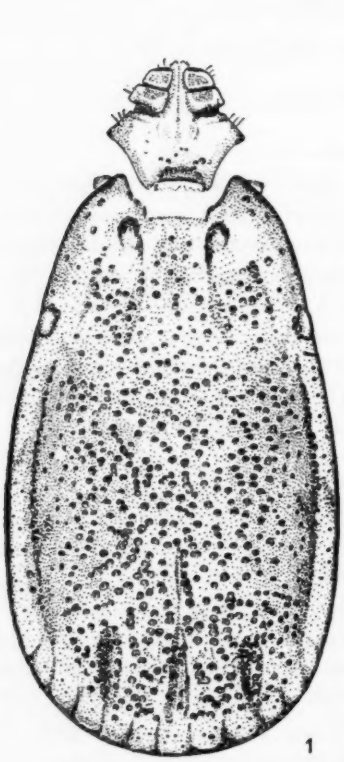
The *rostrum* is slightly broader than it is long and the palps are short.

The *basis capituli* is almost twice as broad as it is long; its postero-lateral border is deeply concave and sweeps backwards; it is twice as long as the short, straight, or slightly convex antero-lateral border. The auriculae are anterior and acute. The antero-lateral border of the basis capituli is usually interrupted by a shallow groove running close to the anterior border parallel with palpal segment I. The posterior border of the basis capituli is straight or slightly concave, with well-developed triangular cornuae, which end acutely or subacutely. The dorsal surface immediately anterior to the posterior border with an elliptical ridge concave posteriorly, which appears in many specimens to join the cornuae, the area between this ridge and the posterior border being slightly depressed. Cornual ridges not strongly developed, so that the median field is only slightly depressed with few large punctations. Two or three discrete submedian punctations may impinge on the secondary elliptical ridge mentioned above, representing *areae porosae* of the female.

A small, highly-chitinized subcollare present. *Palps* short with article I visible dorsally. Article II twice as broad as long, with the external lateral border shorter than the internal border and with a chitinized, upturned, posterior border. Article III also broader than long, with its anterior border rounded and chitinized, giving a blunt appearance to the palps. Dorsal surface of both articles carry a shallow concavity. *Conscutum* inornate, glossy, dark-brown, convex, elongated, narrowing slightly at eye level, widest immediately anterior to the spiracles.

* Now Director of Veterinary Services, Kaduna, Nigeria.

Projection of coxa I only slightly visible dorsally. Eyes yellowish, oval, flat, or very slightly convex, and limited dorsally and internally by three to four somewhat shallow punctations some of which may coalesce. Conscutum covered with numerous punctations of medium size, which tend, centrally and in front of the posterior grooves, to be arranged in lines, leaving small irregular shagreened areas.



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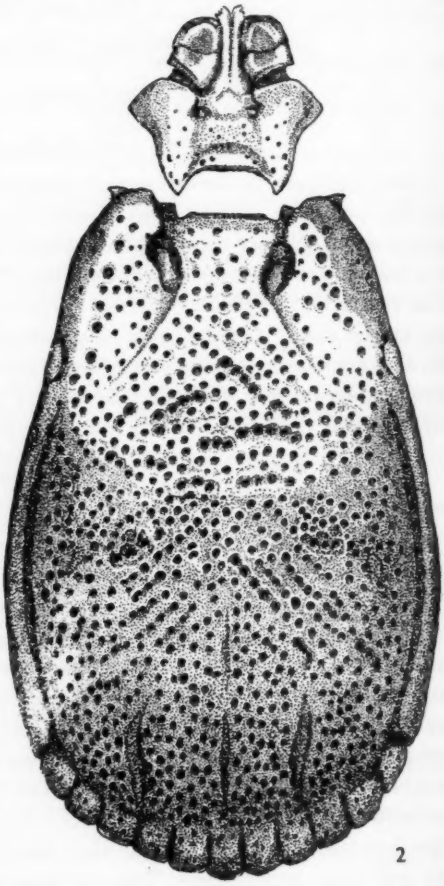

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Fig. 1. *R. hurti* n.sp. Dorsal aspect of male. Flow of posterior grooves may be more coarsely rugose than shown above.

Fig. 2. Dorsal aspect of male.

The punctations themselves appear to be directed inwards at an angle to the scutal surface, having a deeper anterior portion with a superficial posterior portion. This may give some of the punctations a slightly elongated and unequal appearance; and on microscopic examination they may vary in size with the depth of focus. The punctations may coalesce, so that two or three share a common depression. These common depressions often, on cursory examination, give the appearance of large punctations. A few apparently genuine large punctations may, however,

occur, especially in the median cervical region. Punctations are more numerous and the tendency to coalesce more evident immediately in front of festoons, but they are discrete on the festoons. In many specimens the female scutum appears to be represented, being limited by a semicircular line of punctations starting behind the eyes. A few large punctations occur on the shoulder tips, but clear,

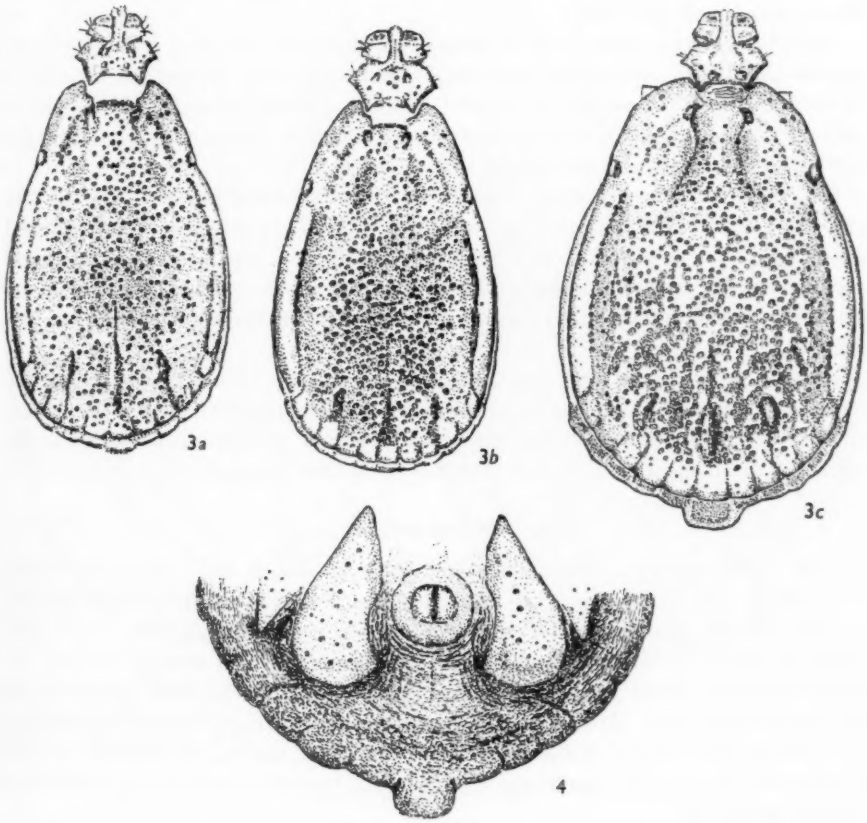

Fig. 3. *R. hurti* n.sp. Dorsal aspect of three males showing range of variations in punctations and shape. (*a*) Sparse punctation, (*b*) typical pattern of punctations, and (*c*) dense punctations and oval shape.

Fig. 4. Anal plates—chitinized scutes on ventral festoons not shown.

glossy areas with a few fine punctations are found anterior and immediately dorsal to the eyes. On either side of the conscutum adjacent to the marginal grooves there may be an area relatively free of medium punctations, but with very fine punctations; this area may be present also in *Rhipicephalus appendiculatus*, and in *R. ziemanni*; it may be bounded dorsally and mesially by a fine ridge. Punctations are absent on the marginal folds and they are few, fine and discrete on the festoons.

Cervical pits deep and convergent, being deepest anteriorly and mesially; they may continue to the level of the eyes as shallow, divergent, linear cervical grooves. The *lateral grooves* are represented by five to six large punctations running from the external edge of the cervical pits backwards to the level of the eyes. The lateral and cervical grooves bound small, depressed, divergent cervical areas, which are relatively clear of punctations. These characters are, however, not readily seen in all specimens.

Marginal grooves shallow immediately posterior to the eyes, but they soon deepen and carry numerous coalescing punctations and extend backwards to include the first and rarely the second festoon. Strictly speaking, however, no definite grooves exist, the external margin only having a clear cut edge where the surface of the 'groove' rises steeply to the lateral fold.

Posterior grooves well defined, especially the paramedian grooves. The median groove is linear, shallow and narrow anteriorly, but it may have a wider and deeper posterior portion in front of the median festoon. The paramedian grooves are semicircular, or bow-shaped, elongated furrows, which converge posteriorly and end between the 3rd and 4th festoon. The floor of all three grooves is coarsely rugose.

Festoons well defined, the first festoon being broader than it is long; but the remaining festoons, including the median festoon, are longer than they are broad. In engorged specimens the body extends beyond the conscutum posterior to the spiracles, often showing a distinct bulge opposite the 1st and 2nd festoon.

Ventral surface

Anal plates strongly developed. They may be raised from the surrounding integument (Fig. 4). They are broadly triangular, external border being relatively straight and the internal border concave along the posterior half of its length. The posterior border is rounded and curved and bends forward sharply to join the internal margin. There is usually a blunt, chitinized point where the posterior and internal borders meet. Accessory anal plates, lightly chitinized and ending bluntly, are present. The ventral festoons have well-chitinized scutes; the median festoon protrusible. The caudal appendage is large and is broader than it is long when it is viewed ventrally.

Legs subequal; the fourth pair of legs not markedly longer than first three pairs.

FEMALE (Figs. 5, 6)

Salient features. A medium-sized tick with scutum oval, heavily chitinized, broader than long. The cervical pits and cervical and lateral grooves are present, the lateral grooves reaching the posterior border. The central scutal area is covered with dense, medium-sized, fairly uniform punctations, while the lateral borders are raised and glossy and carry few fine punctations, except on the shoulders. The *basis capituli* is broad, the auriculae acute and only slightly anterior, and the palps are longer than those of the male.

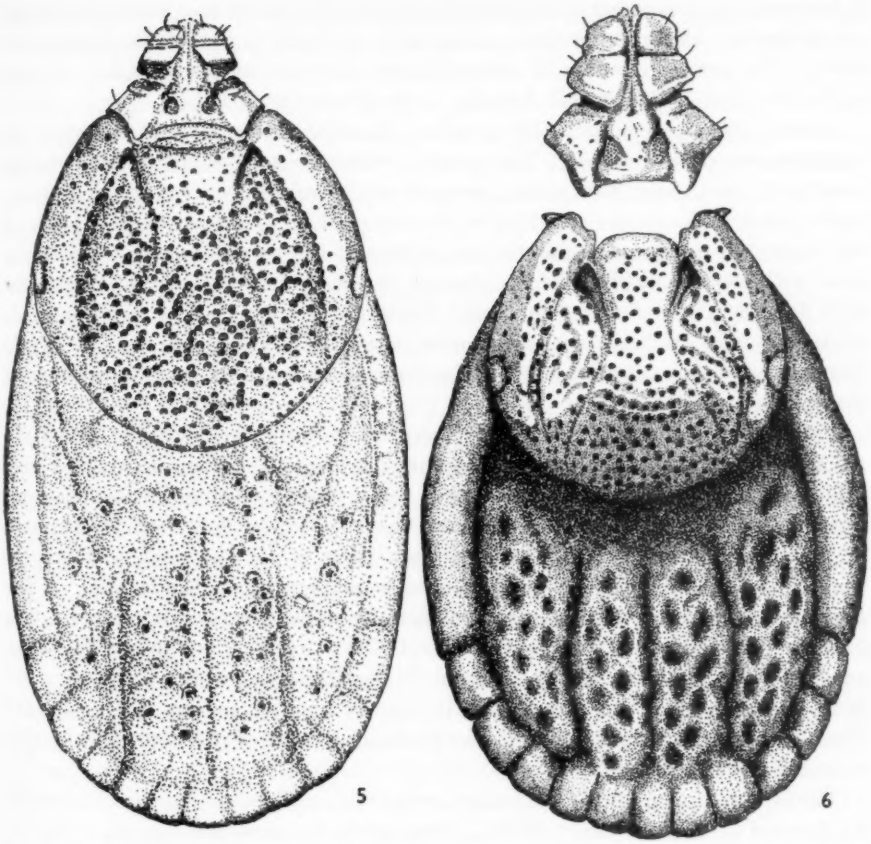

Fig. 5. *R. hurti* n.sp. Dorsal aspect of female. Rostrum is slightly bent ventrally making basis appear shorter than it really is.
Fig. 6. Dorsal aspect of female.

Detailed description

Rostrum one and a half times as broad as long; palps short; auriculae acute.

Basis capituli twice as broad as it is long, with the concave postero-lateral border only slightly longer than the straight antero-lateral border, so that the auriculae are median or only slightly anterior; they are acute and are directed backwards. The posterior border of the basis is straight and carries short blunt cornuae. Cornual ridges are well developed, so that the median field of the basis is depressed.

The *areae porosae* are large and oval, with the elongated diameter antero-posterior and placed about one diameter apart; each area may, or may not, have a short anterior gutter.

Palps longer than those of the male, article II being broader than it is long and the short external border like that of the male. Article III is about as broad as it

is long and has a rounded, blunt anterior border. The margins of both articles are not so heavily chitinized as those of the male are, and they have no dorsal concavity. The posterior border of article II may, like that of the male, be strongly chitinized. Article I is visible dorsally. *Subcollare* present, broad.

Scutum broadly oval, with the greatest diameter at the level of the eyes, its emargination deep and wide. The *cervical grooves* start as elongated, converging deep pits, continuing backwards as shallow, diverging grooves, which become indistinct in the posterior one-third of the scutum. The *lateral grooves*, like those of the male, are not proper grooves, the surface of the so-called grooves being on level with the cervical field of the scutum, the sharp internal edge of the lateral fold defining the exterior limit only. These grooves or furrows bend inwards posteriorly and reach the posterior border, the internal margin of the lateral fold being steepest opposite the level of the eyes; large punctations impinge on this lateral border of the furrow.

The areas between the lateral and cervical grooves are depressed and have a somewhat striated appearance. The central field of the scutum is covered with punctations of medium size, which tend to be fewer and finer in the posterior area, along the cervical grooves and near the posterior border. Occasionally two or more punctations may be confluent, but there is not the same tendency as there is in the male for the punctations to be arranged in linear fashion. A few large punctations occur in the central field between the cervical grooves. The raised, glossy edges of the scutum are relatively free of punctations and such punctations as do occur are fine, except on the shoulders, where there are a few large punctations. Eyes large, slightly convex and, as in the male, bounded dorsally by a row of punctations. The posterior border of the scutum is bluntly rounded and slightly sinuous.

The type specimens, male and female, were from a collection taken from a buffalo by Lieut.-Col. R. A. F. Hurt, D.S.O., after whom the species is named, at Karati Forest Naivasha, Kenya (Coll. No. 221). These specimens have been deposited in the British Museum. Paratype specimens have been sent to Dr Gertud Theiler, Veterinary Laboratory, Onderstepoort, South Africa, and to Dr Harry Hoogstraal, Naval Medical Research Unit, Cairo, and to Dr J. A. Travassos Santos Dias, Mocambique, all of whom have confirmed that the species is new.

DISCUSSION AND DIAGNOSIS

The commonest variations of *R. hurti* are in the shape of the conscutum and in the depth and the density of punctations (Fig. 3). The normal measurements of the conscutum are 3×2 mm., these measurements being almost identical with those of *Rhipicephalus neavei*. In some individuals, however, the conscutum appears abnormally elongated, with measurements of $3{\cdot}5 \times 2$ mm., while in others the posterior portion is broad and oval, being about 2·5 mm. broad.

The punctations in typical specimens are dense, especially in the posterior half of the conscutum. In some specimens, however, the punctations appear shallow and discrete and fewer in number and they remain discrete even in front of the festoons (Fig. 3*a*). In other specimens, especially the oval variety (Fig. 3*c*),

punctations are more numerous than they are in the typical forms and they tend to coalesce more than usual in front of the festoons and between the three posterior grooves, giving a corrugated effect to this portion of the conscutum.

The male *R. hurti* can readily be distinguished from *R. capensis* by the fact that the male *R. capensis* has a definite marginal groove, the punctations are more dense and are uniformly distributed and generally discrete. The basis capituli of *R. capensis* is more thickly set (index 0·5) than in *R. hurti* (index 0·2). The two species also differ in colour and size.

The female *R. capensis* has a more oval scutum, the punctations differ as those of the male do and the marginal grooves are also more definite.

R. hurti has also been compared with *R. serranoi* Dias 1950. Dr G. Theiler, who has examined specimens of both species, regards the punctations in the male *R. serranoi* as being coarser and more evenly distributed. The internal edge of the lateral fold is possibly steeper and gives a more definite appearance of a marginal groove; anteriorly there is no row of punctations in place of a lateral groove; the posterior grooves are irregular depressions rather than definite grooves. The punctations on the lateral fold, although they are smaller than they are in the central field, are not so small as those of *R. hurti*. In the female *R. serranoi* the coarser punctations are again more evenly distributed and reach to the edge of the scutum. The lateral fold in the females of both species is but poorly developed. In *R. hurti* it is edged by an indistinct row of punctations; in *R. serranoi* there is no lateral fold at all.

R. hurti is very often associated in Kenya with *R. jeanneli* Neumann 1913 (Zumpt, 1943) and, because the range of variation of both species is as yet unknown, it is usually difficult to distinguish between these two species. The male *R. jeanneli*, however, is less heavily chitinized and is lighter brown than *R. hurti*; its punctations are never so dense, especially on the posterior half of the scutum and the marginal and dorsal grooves are less distinct. The punctations on the female *R. jeanneli* give a more coarse appearance to the scutum and they extend on to the lateral areas of the scutum; the lateral grooves are even less definite and the scutum is lighter brown and relatively broader.

HOSTS AND GEOGRAPHICAL DISTRIBUTION

Most of the present material, including the type specimens, was collected from buffalo in various localities in the Rift Valley, north and west of Naivasha; especially in the Kipipiri area and at Elementeita.

Specimens deposited in the British Museum by Dr E. A. Lewis and identified as *R. capensis* from cattle and dog from Njoro and Naivasha and from bush-buck and buffalo from Karati forest are now identified as *R. hurti*. Others in the same collection identified as *R. bursa* from bush-buck and buffalo are also *R. hurti*. The present known distribution of this species is therefore in the Rift Valley of Kenya in and around Naivasha at a height of approximately 6000 ft.

SUMMARY

A new species of Rhipicephalid tick, *Rhipicephalus hurti*, is described from game animals from the Rift Valley of Kenya. The new species is compared with *R. capensis*, *R. serranoi* and *R. jeanneli*.

My thanks are due to Dr G. Theiler, Onderstepoort, who confirmed that *R. hurti* could be correctly regarded as a new species and has been most generous in her assistance. My thanks are also due to Dr Santos Dias and Dr Hoogstraal for their most helpful criticisms; to Lieut.-Col. Hurt for his interest and care in sending me numerous tick-collections from the Naivasha area of Kenya; to Mr Alex Wiley and Mr Guggesburg for their careful drawings; and to Miss Walker, E.A.V.R.O., and my colleague Mr Pollard, for their generous help at all times.

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THE LIFE CYCLE OF *PARAMPHISTOMUM MICROBOTHRIUM* FISCHOEDER, 1901 (TREMATODA, PARAMPHISTOMIDAE)

By J. A. DINNIK and N. N. DINNIK

East African Veterinary Research Organization, Muguga, Kenya

(With 6 Figures in the Text)

CONTENTS

	PAGE
Introduction	285
Material and methods	285
Egg and miracidium	286
Development in an intermediate host	287
(*a*) Sporocyst	287
(*b*) Rediae	288
(*c*) Cercaria	292
Development in a final host	296
Summary	298
References	299

INTRODUCTION

Paramphistomum microbothrium was first described briefly by Fischoeder in 1901. Later, in 1903, he again described the species in much greater detail. The material used for description of the species consisted of fourteen specimens, collected from *Gazella dorcas* of North Africa. Maplestone (1923) considered *Paramphistomum microbothrium* Fischoeder, 1901, to be synonymous with *Paramphistomum cervi* (Zeder, 1790), and referred nineteen other lots of paramphistomes, collected from different parts of Africa, including the Sudan and Kenya Colony, to the latter species. Näsmark (1937) re-established *P. microbothrium* Fischoeder as a valid species after re-examination of Fischoeder's original material and studying specimens of this species collected by the Swedish Sudan Expedition at Cairo. Nineteen series of microscopical sections of *P. microbothrium* found in the collections of the late Prof. A. Looss were also examined.

P. microbothrium is a common parasite of cattle in the Kenya Highlands. This species was erroneously considered by the writer to be *P. cervi*, when the intermediate snail host, *Bulinus alluaudi* (Dautzenberg), was first determined in Kenya (Dinnik, 1951).

In the present investigation a number of experiments were carried out to determine the life history of *Paramphistomum microbothrium* Fischoeder, 1901, found in Kenya.

MATERIAL AND METHODS

In the observations recorded here on the life history of *P. microbothrium*, flukes collected from cattle slaughtered at the abattoirs of Nairobi and Nakuru in Kenya were used. Eggs were obtained by placing adult flukes in 0·9 % salt solution in an incubator at a temperature of about 37° C., where they would deposit eggs for

about 12 hr. The flukes were removed before they died and some of these, as well as fresh flukes taken from the same animal, were preserved in alcohol in order to confirm by examination of sagittal sections, the preliminary identification of the living flukes.

The eggs deposited by the flukes were washed in several changes of water, to remove surrounding debris, and were then incubated in tap water until the miracidia hatched.

The snails used in these experiments were laboratory-reared specimens of *Bulinus alluaudi* (Dautzenberg, 1908), a species occurring commonly in Kenya and closely related to *B. truncatus* Aud. of Egypt.*

Attempts to infect snails experimentally were made by placing them in a glass basin containing water, to which numerous freshly hatched miracidia were then added. After being left in the basin for 4 hr. the snails were transferred to an aquarium, where they were kept under observation at a temperature of between 18 and 22° C.

Altogether, during 1950–2, fifteen series of snails, each of which comprised over a hundred snails, were infected experimentally to provide material for this study of the life history of *Paramphistomum microbothrium*.

Sporocysts, rediae, and developing cercariae were dissected out from the snails and studied while alive, either unstained or stained with vital stains only. Young and adult flukes from experimentally infected cattle and goats were fixed in 70% alcohol and stained with alum carmin for examination *in toto*, or embedded in paraffin wax, cut into sagittal sections and stained with haemotoxylin and eosin.

EGG AND MIRACIDIUM

The eggs of *P. microbothrium* are large, measuring from 160 to 184μ long by 89 to 103μ wide. The egg-shell is about 3·5μ thick and has an easily recognizable operculum about 30μ in diameter. When deposited each egg contains an embryo, the outline of which is seen among yolk cells in the centre of the egg. The embryo is at an early stage of cleavage, consisting of three to five cells.

Miracidia hatched from the eggs on the 14th to 16th day when kept in water at a temperature of 26–28° C. When hatching began, large numbers of miracidia emerged from the eggs within a short period, the water in the glass vessel soon containing great numbers of swimming miracidia.

The size of the miracidia vary from 228 to 294μ long and from 68 to 81μ wide. The body is covered with twenty ciliated epithelial cells, arranged in five transverse rows. The first, or anterior, row comprises six cells, each wide at the posterior border and narrowing gradually toward the anterior border. The second row has eight long rectangular cells. The third row comprises four rectangular cells, much broader and shorter than those of the second row. Two cells cover the caudal extremity. The central cavity of the miracidium contains germinal tissue with large

* The authors are indebted to Prof. J. Bequaert of the Museum of Comparative Zoology, Harvard College, U.S.A., for the identification of the snails used in our experiments. *B. syngenes* Preston, 1913 (described from Lake Naivasha, Kenya) is considered as a synonym of *B. alluaudi* (Dautzenberg, 1908), the species originally described from Nairobi, Kenya.

nuclei (the number of the nuclei is about fifteen), several germinal cells and a few germinal balls.

After hatching the miracidia swam very actively, generally moving in a straight line and only changing direction on reaching the water surface or the vessel wall. When a snail, *Bulinus alluaudi*, was placed in water containing several miracidia they quickly concentrated around it. One after the other, the miracidia soon entered the respiratory cavity of the mollusc and did not reappear. A miracidium was often seen to approach the snail repeatedly, touch its outer soft part, and swim away, returning later and disappearing into the respiratory cavity. Usually all miracidia entered the snail during the first hour. If no host was available, most of the miracidia became exhausted by ceaseless swimming, and died within 24 hr. Only a few survived some hours longer.

DEVELOPMENT IN AN INTERMEDIATE HOST

(*a*) *Sporocyst*

The earliest sporocysts seen were found in a snail dissected on the 11th day after exposure to miracidia. Nothing was learned, therefore, of the development of this trematode, during the first 10 days in the intermediate host.

Usually from one to four sporocysts developed after exposure to miracidia—a very small number in comparison with the large number of miracidia used for infection of the snail. When snails were exposed to many thousands of miracidia, from eight to thirteen sporocysts were recovered, but the majority of such heavily infected snails soon died.

In most snails the sporocysts were situated in the tissue of the respiratory cavity; in some they were found in the body spaces surrounding the intestine; rarely they were found in the headfoot. In one snail exposed to heavy infection, three sporocysts containing rediae were found in the liver.

The youngest sporocyst examined was 0·32 mm. long, its body cavity containing only germinal cells and a few small embryo-balls. Fully developed sporocysts were up to 3·6 mm. long, the elongated body being divided into parts, by a varying number of deep constrictions, each part containing embryo-balls and developing rediae (Fig. 1).

There was much variation in the rate of development of the sporocysts. In a snail examined 11 days after infection one young sporocyst containing many embryo-balls and one redia were found. Another snail dissected 12 days later contained three sporocysts, of which two were very small and oval, with germinal cells in the body cavity only, and the third showed the long constricted body, containing germinal cells and embryo-balls.

Usually, however, after 15 days of development in the snail, the sporocysts contained from five to ten rediae and many embryo-balls in the body cavity. Thereafter the number of rediae increased, and by the 19th day the sporocyst had as many as twenty-eight rediae and over twenty embryo-balls. It appeared that within 25 days the sporocyst completed its development and died after producing many rediae. In heavily infected snails, however, the rate of development was retarded. It is difficult to determine the number of rediae produced. In one

snail, eleven sporocysts containing rediae were found on the 35th day after infection. One of these sporocysts contained twenty-three rediae and seventeen developing embryo-balls.

In our experiments no evidence was found of the production of daughter sporocysts by the sporocysts of *P. microbothrium*.

(*b*) *Rediae*

The first young rediae to emerge from a sporocyst were observed on the 14th day after infection of the snail. At the time of liberation young rediae measured from 0·22 to 0·35 mm. long and already showed most of the structures of the mature redia. They had developed a pharynx and a rhabdocoel intestine extending to the posterior half of the body. The body cavity contained from five to ten germ and embryo-balls, but no birth pore was visible in small, young rediae.

After emerging from the sporocyst the young rediae migrate into the liver of the snail, where further development proceeds. Growth of the rediae is rapid and, as they increase in size, the germinal material of the body cavity develops rapidly. The germinal masses attached to the posterior end of the body cavity produce the germinal cells which develop into embryos, which are at first small and consist of only a few cells. The cells multiply and the embryos grow into embryo-balls, which gradually fill the greatly enlarged body cavity of the redia. The smallest embryo-balls are situated in the posterior part of the body cavity, the size of the balls increasing towards the anterior part of the body cavity of the mother redia. At the beginning of the period of multiplication, a redia of the first generation produces only rediae of the second generation.

The youngest daughter redia found in a first-generation redia was seen on the 20th day after the exposure of the snail to the miracidia. This snail contained one sporocyst full of young rediae, and many free rediae in various stages of growth. Only one of these rediae had a clearly recognizable daughter redia in its body cavity. The remaining rediae contained embryo-balls in which the organs were still unrecognizable. In other experiments daughter rediae were usually found in the first-generation redia from 25 to 36 days after the infection of the snails.

The mature rediae measured from 0·55 to 0·75 mm. long and from 0·17 to 0·22 mm. wide. The diameter of the pharynx in the redia varied from 0·046 to 0·049 mm., and the length of the sac-shaped intestine was from 0·15 to 0·22 mm. The birth pore was clearly visible and was situated at the level of the posterior half of the intestine. No lateral appendages were present in the redia of *P. microbothrium*.

Mother rediae usually contained from one to three, rarely more, daughter rediae lying in the anterior part of the body-cavity, and from fifteen to twenty-five embryo-balls in its posterior part (Fig. 2). In one out of hundreds of rediae examined seven daughter rediae and eighteen embryo-balls in the mother redia were found. After the structures have become differentiated, the daughter redia begins to move and leaves the mother redia by the birth pore. The body length of the rediae just emerged from the mother redia varied from 0·22 to 0·35 mm. No morphological difference was found between the young rediae of the first generation and those of the second generation.

From the 28th day onwards, young rediae and young cercariae were observed in the body cavities of some parent rediae. At this stage the parent redia generally contained from one to three daughter rediae in the anterior part of the body cavity, with one or two developing cercariae lying behind them, and about fifteen to eighteen embryo-balls filling the rest of the body cavity (Fig. 3). Both daughter rediae and cercariae in the same parent redia were seen during a short period after which the rediae of the first generation ceased to develop young rediae and produced cercariae only. In general appearance, shape and size the parent rediae remained the same. The germinal masses attached at the posterior end of the body cavity continued to produce a steady stream of free embryos which, in various stages of growth, filled the body cavity; but at this phase of the redia life the embryo developed into cercariae only.

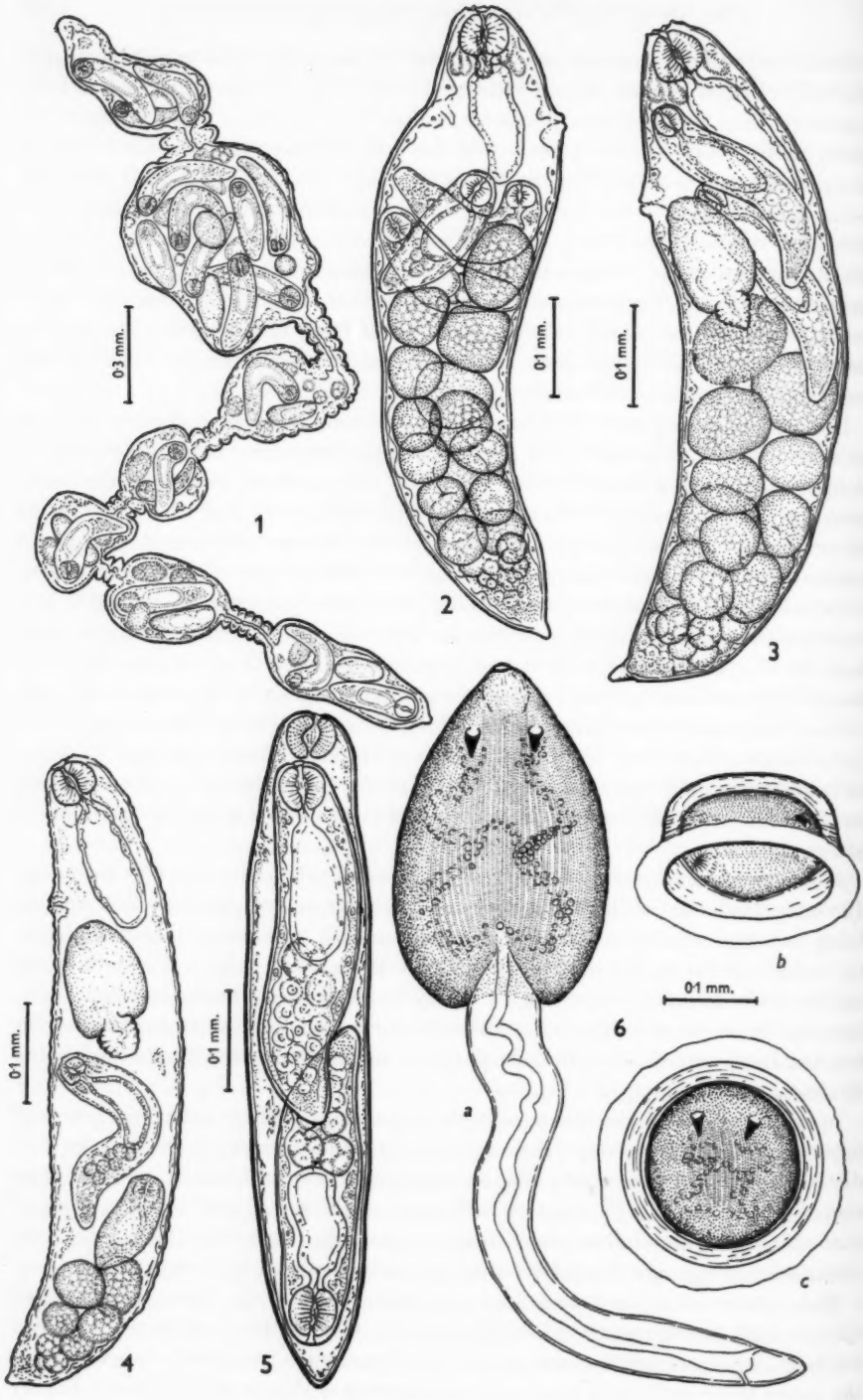
During the development of embryo-balls into cercaria, some of the cells become organized into the sucker, and the oesophagus becomes visible. The embryo elongates and the rudimentary tail develops. From one to five such developing cercariae can be seen in the anterior part of the body cavity of the redia producing cercariae only. Usually no eye spots are visible in the cercariae developing in the parent redia. However, in some old rediae, in which the rate of production of new embryo-balls had decreased considerably and the emergence of cercariae was therefore somewhat delayed, cercariae having two clearly recognizable eye spots were found. As soon as the immature cercariae are able to move they leave the parent redia and continue their development independently in the liver of the snail.

The redia produces cercariae during a long period, lasting at least 30 days, this period representing the peak of productivity of the larval development of *P. microbothrium* in the snail host. As a result of this productivity the liver of the snail soon becomes infested with numerous free cercariae in successive stages of development and growth.

After this peak of productivity has been reached, the redia becomes senescent. The body of the old redia is dirty greyish in colour, almost opaque, the contents being no longer clearly visible. The germinal masses, exhausted during the life of the redia, are no longer present at the end of its body cavity. The number of embryo-balls decreases and the body cavity becomes empty as the last developing cercariae leave the parent redia. As the life of the redia has drawn to its close, the few remaining embryo-balls in its body cavity no longer develop into cercariae, but into daughter rediae again (Fig. 4).

In our experiments, the first aged redia containing daughter rediae was observed in the snail on the 62nd day following infection. Later, from the 70th to the 80th day of infection, several aged rediae were readily found in the snail. Some of these contained one cercaria, the last to be formed in the redia, and, behind it, one or two young daughter rediae and a few embryo-balls. But more frequently aged rediae containing only daughter rediae and a few embryo-balls were found.

The mother redia dies soon after the completion of the development of all embryo-balls into daughter rediae. We frequently found dead rediae, whose shapeless body still contained from two to five daughter rediae, which were late in leaving the parent body cavity and continued their growth within it after its death (Fig. 5).



Figs. 1—6 (see opposite for legends)

We could not find any difference between the daughter rediae formed in the mother redia at the beginning of its life and the daughter rediae developed at the end of it.

The development of second-generation rediae was identical to that of first generation rediae. At the beginning of their period of multiplication they produced daughter rediae, then numerous cercariae, and finally, rediae again.

In snails dissected 50–60 days after infection we observed rediae of the second generation which contained daughter rediae, some also contained a cercaria behind the rediae, while the rediae of the first generation were producing cercariae only. At this stage the liver of the snail showed numerous developing free cercariae and fully developed cercariae ready to escape.

When the first-generation rediae reached the last phase of their life, three generations of mature rediae were observed in the snail: aged first-generation rediae with a few daughter rediae in their almost empty body cavities, second-generation rediae at the peak of cercaria production, and third-generation rediae starting to form fourth-generation daughter rediae. When this stage was reached, the snail harboured young rediae of various sizes, escaped from parent rediae of the first, second and third generations, as well as numerous developing and developed cercariae.

Among the Paramphistomidae daughter rediae within a parent redia were described by Looss (1896) in *Amphistomum conicum* R. of Egypt. This fluke was most probably not *Paramphistomum cervi* (Zeder, 1790) but *P. microbothrium* Fisch., 1901, as has been suggested by Näsmark (1937). Le Roux (1930) mentions that daughter rediae occur in the life cycle of *Cotylophoron cotylophorum* of South Africa, and Bennet (1936) found three well-developed and three developing rediae within the rediae of a species which was identified as *C. cotylophorum*, but which Price & McIntosh (1944) consider to be a new species of the genus *Paramphistomum*, namely *P. microbothrioides*. Buckley (1939) found rediae containing either immature cercariae or daughter rediae in the development of *Cercaria fraseri*, an amphistome cercaria from *Planobris exustus* in Assam.

So far as we could find, rediae and cercariae occurring together within a parent redia have only been observed in *Fasciola hepatica*, by Thomas (1883) and Krull (1933), in *Gastrodiscus aegyptiacus* by Looss (1896), and in *Clinostomum marginatum* by Edney (1950). The latter author found rediae of *C. marginatum* of different stages of development in wild snails, *Helisoma trivolvis*, which were 2 or 3 years old. Some of these rediae contained both daughter rediae and cercariae. Wild snails

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Fig. 1. Sporocyst of *P. microbothrium*.

Fig. 2. Redia of the first generation with young daughter rediae, 30 days after exposure of the snail to miracidia.

Fig. 3. Redia of the first generation with two daughter rediae and one developing cercaria, 42 days after exposure of the snail to miracidia.

Fig. 4. Redia of the first generation at the close of its life end and productivity, 74 days after exposure of the snail to miracidia.

Fig. 5. Dead redia of the first generation with two daughter rediae which delayed in the parent body cavity, 76 days after exposure of the snail to miracidia.

Fig. 6. Mature cercaria and encysted metacercaria of *P. microbothrium*.

(1 and 2 years old) could not be experimentally infected with *Clinostomum marginatum*, while laboratory-reared snails of the same species, 2 months old, were easily infected in the laboratory and, when examined 60–70 days later, contained rediae, in some of which both cercariae and rediae were seen. These observations led Edney to conclude that continuous production of rediae by rediae can maintain the infection in the snail into the 3rd year.

In our experiments with *Paramphistomum microbothrium* we were able to trace quite definitely four redial generations, which develop within the first 80 days after the penetration of miracidia into the snail. In snails dissected thereafter rediae in different stages of development were found, but we could not detect any morphological features to permit distinction between the different overlapping generations of rediae at that stage. The results of dissections of snails infected for over a 100 days, in which the number of rediae at different stages of development were counted, were as follows.

Two snails were dissected 135 and 136 days respectively after exposure to miracidia. In the first, there were found fifty-five small young rediae, 127 young rediae with embryo-balls only in the body cavity, ten rediae containing daughter rediae and embryo-balls, sixty-five rediae containing only cercariae and embryo-balls, nine aged rediae with daughter rediae inside, and 417 developing and developed free cercariae. The other snail contained 186 small young rediae, 395 rediae with embryo-balls only, nineteen rediae containing daughter rediae and embryo-balls, 185 rediae containing developing cercariae and embryo-balls, ten aged rediae with daughter rediae and 760 developing and developed free cercariae.

In a snail dissected on the 209th day after infection, there were found 212 small young rediae with embryo-balls only in the body cavities, eleven young rediae containing daughter rediae, 112 mature rediae producing cercariae only, three aged rediae with daughter rediae, and numerous developing and developed cercariae.

In a snail examined 392 days after exposure to miracidia, there were found many rediae in various stages of development, whose number was not counted, and 1839 developing and developed free cercariae.

Two snails were dissected on the 431st day: one contained forty small young rediae, 169 rediae with embryo-balls only, thirty rediae with cercariae and 433 developing and developed free cercariae; the other snail harboured 206 small young rediae, 255 rediae with embryo-balls only, five rediae with daughter rediae and embryo-balls in the body cavities, 107 rediae with developing cercariae, and 254 developing and developed free cercariae.

One snail lived for as long as 504 days after infection with miracidia of *P. microbothrium*. When this snail was examined, it proved not to be heavily infected. It harboured seventy small young rediae, fifty-four rediae producing cercariae, one old redia with a daughter redia in its body cavity, and 873 developing and developed free cercaria.

(c) *Cercaria*

In this series of experiments, the first cercariae which emerged from parent rediae were observed 30 days after exposure of the snail to miracidia.

Cercariae emerge from the redia at a very immature stage. The body length

varies from 0·13 to 0·15 mm., or about one-third of the size of the mature cercaria. The mouth of the young developing cercaria is open and encircled by oral papillae. The oral sucker is easily recognizable, and the oesophagus and gut caeca are visible. Shortly after birth, the eye spots become prominent. The tail of the young cercaria is broad and very short, about half of the body length. The two lateral ducts of the excretory system join the tail duct in the median dorsal line of the body. The newly born cercaria is only capable of very weak movement. Further development of cercariae takes place in the liver tissue of the snail.

Numerous cercariae at different stages of development were present in the liver of snails dissected on the 42nd day and thereafter. This provided the opportunity of studying their development to maturity, which follows the pattern described by Bennett (1936) for *P. microbothrioides* and by Brumpt (1936) for *P. cervi*.

The acetabulum first becomes clearly visible when the cercaria is approximately 0·20 mm. in length and a lumen becomes conspicuous in the acetabulum when the cercaria has reached a size of 0·30 by 0·15 mm. At this stage the two lateral excretory ducts with their diverticula, and the cross-anastomosis situated at the middle of the body are clearly recognizable, although they are not yet filled with excretory granules. The cystogenous glands, which increase in number as the cercaria develops, begin to produce granules and rods. These granules and rods increase rapidly so that in cercariae of more than 0·30 mm. in length, the cystogenous cells are packed with them. Pigment starts to grow out from the eye spots and, simultaneously with the formation of cystogenous granules and rods, the pigment spreads in irregular dendritic branches over the surface of the cercaria. In the mature cercaria this pigment, dark brown in colour, is distributed over the entire body as a solid layer. This heavy pigmentation, together with the cystogenous rods and granules, makes the body opaque, obscuring the internal structure of the cercaria. Subsequently the eyes, which retain their pigmentation, are formed. The tail becomes greater in length than in width, and its length increases rapidly during the process of pigmentation of the cercaria. The tail itself remains free of pigment and transparent.

In our experiments mature cercariae began to emerge from the snails on the 43rd–46th day after exposure to miracidia. It appears, therefore, that the cercaria requires about 14 days to develop after leaving the parent redia.

The body of the mature cercaria when relaxed is about 0·40 mm. long, by 0·25 mm. wide. The tail is about twice the length of the body. The living cercaria can extend and contract the body and tail considerably. The body of swimming cercariae becomes almost spherical, 0·22 by 0·28 mm., while the tail extends to 0·80 mm., in length. On the other hand, a crawling cercaria can expand its body to 0·58 mm. and contract its tail to 0·40 mm.

The body of the mature cercaria is heavily pigmented and there is a profuse supply of rods in the cystogenous cells which, together with the pigmentation, makes the body opaque. The surface of the body is covered with fine longitudinal striations. Two eyes are situated immediately posterior to the oral sucker. They are conical in shape with the base surmounted by a refractile lens directed anteriorly. Eye cone is 30μ long and 20μ in diameter at its base.

The acetabulum, located on the ventral surface of the posterior end of the body, is highly muscular and measures 114–137μ in diameter. Oral sucker is from 68 to 82μ in diameter. The extent of oesophagus and caeca cannot be determined in living mature cercariae. The main excretory ducts can be traced in living cercariae because they are filled with excretory granules varying from 4 to 15μ in diameter. Two large excretory ducts join the bladder at the acetabulum. From this point they pass outward and forward for a short distance and then turn inward. At the middle of the body an arch-shaped anastomosis lies across the body. Anterior to this cross-connexion the main ducts turn outward and then inward again posterior to the eyes. They pass forward ventral to the eyes and reach the sides of the oral sucker. Short diverticula are formed on the ducts immediately posterior to the eyes. Excretory duct of the bladder is joined by the caudal excretory tube which passes backward through the centre of the tail and broadens to form a small bladder at the posterior end of the tail. From this broadening a short duct passes laterally on each side to open to the exterior. Excretory duct of bladder and caudal tube contain no granules (Fig. 6*a*).

After emerging from the snail cercariae swim actively and congregate near the surface of the water where light is most intense. The cercariae continue swimming for about half an hour, and then attach themselves to vegetation or to the wall of the glass vessel and begin to encyst. First the cercaria forms a thin membranous shell surrounding the body with a base which fastens the shell to the substratum. The cercaria then liberates the cystogenous material from the body, and by moving within the membranous shell spreads the cystogenous substance evenly to form the thick wall of the cyst. The tail of the cercaria becomes released from the body but usually remains attached to the cyst wall for some time, continuing to move vigorously.

The fully formed cyst is round when viewed from above, and dome-shaped in lateral view. The slightly flared base acts as a support for the cyst and attaches it to the substratum. The diameter of the cyst varies from 200 to 250μ and the diameter of its base from 245 to 310μ. The total height of the cyst is about two-thirds of the diameter. The thickness of the wall is about 25μ. The cyst wall is colourless and transparent, but the metacercaria retains its heavy pigmentation and, therefore, cysts look like black spots when they are examined on a light background and appear grey with pearly lustre when they are seen on a dark background. The metacercaria is situated in the cyst with its dorsal surface upwards. Owing to the pigmentation the only structures visible are the oral sucker, acetabulum, eyes, main ducts of the excretory system filled with granules, excretory pore, and longitudinal striations on the body (Fig. 6*b* and *c*).

The emergence of cercariae can readily be stimulated by placing the snail in the rays of the sun or of a strong electric light bulb. In one experiment five snails from the same series, each in a glass tube filled with water, were placed under a strong bulb, and at half-hourly intervals the cercariae which had emerged from each snail were counted (Table 1).

The cercariae began emerging from the snails in the first half-hour and continued to emerge in considerable numbers during the two following hours. Soon after that

the snails ceased to shed cercariae. As many as 1412 cercariae emerged from the five snails on the 1st day. On the following day, when these five snails were again placed under the same light, only 586 cercariae were shed.

In a second experiment five snails were taken from the same experimental series and placed into separate beakers. Daily, with the exception of Sunday, each of these snails was removed to a separate tube, placed under an electric bulb for 4 hr., and then returned each to its beaker. The snails withstood this test for 10 days and then died (Table 2).

Table 1. *The number of cercariae shed by five snails during periods of half an hour*

Half an hour ...	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	Total
Snail 1	6	66	97	62	58	7	1	1	0	298
Snail 2	124	100	3	5	8	1	0	0	0	241
Snail 3	2	52	53	39	20	8	4	0	0	178
Snail 4	0	74	111	42	19	2	1	0	0	249
Snail 5	0	36	70	115	177	46	1	1	0	446
Total	132	328	334	263	282	64	7	2	0	1412

Table 2. *The number of cercariae shed daily by five snails*

Days ...	1st	2nd	3rd	4th	5th	6th *	7th	8th	9th	10th	11th
Snail 1	119	352	65	373	41	—	157	72	65	21	Died
Snail 2	29	36	2	15	0	—	0	0	8	Died	—
Snail 3	3	31	11	45	24	—	89	0	69	10	Died
Snail 4	72	311	143	114	98	—	129	0	40	22	Died
Snail 5	29	44	74	27	0	—	0	0	17	Died	—
Total	252	774	295	574	163	—	375	72	199	54	—

* No observation.

The number of cercariae emerging from a snail daily fluctuated considerably and, although more cercariae emerged on the 2nd day, there was a decrease in number on the ensuing days. This decrease was not due to loss of infection of the snail, for dissection showed the liver to be filled with rediae and developing cercariae, but to the fact that the rate of development of new cercariae was insufficient to replace the mature cercariae escaping from the snails. As other experiments have shown, after several days of rest in the aquarium the exhausted snails regained their ability to shed numerous cercariae again.

Infected snails were maintained in aquaria in our laboratory for many months. Most of the snails survived for about 6 months following exposure to miracidia. Emergence of cercariae could at any time be stimulated by placing the snails in direct sunlight or under a strong electric bulb, when numerous cercariae emerged during a short period. Without this artificial stimulation cercariae escaped in much smaller numbers. Dissection of infected snails showed that escaped cercariae were constantly replaced by new generations of young cercariae, so that the liver of the snail contained numerous developing cercariae until the death of the host. In our experiments, the snails began to die in considerable numbers about 6 months after infection. Many infected snails lived for a year but only a few survived longer than a year. It seems that this increase in mortality was not due to infection but was

a natural process, for observations on the life span of *Bulinus truncatulus* made by Barlow & Muench (1951) in Egypt show that the mortality rises sharply when the snails reach the age of 8 months, and none survives over 14 months.

No decrease in the number of cercariae emerging from infected snails was noted during these prolonged observations, although the number shed on different days varied considerably. Eight snails, which had been exposed to miracidia on 20 April 1950, and began shedding cercariae on 3 June, were placed under observation on 10 November 1950. Periodically, until their death, these snails were placed under an electric bulb and the cercariae emerging from each snail were counted.

Table 3. *Emergence of cercariae from eight snails exposed to miracidia on the* 20 *April* 1950

Date	Number of cercariae emerging from each snail							
10. xi. 50	87	181	134	245	137	103	101	492
6. xii. 50	11	215	276	235	172	67	29	318
21. xii. 50	25	117	114	103	102	111	16	Died
6. i. 51	26	72	131	35	149	24	66	—
19. i. 51	17	142	210	108	136	57	34	—
9. ii. 51	27	131	104	211	81	108	86	—
2. iii. 51	46	193	262	230	111	178	Died	—
20. iv. 51	12	253	136	311	270	Died	—	—
14. v. 51	8	120	Died	—	—	—	—	—

Seven of these snails lived until 9 February 1951, five up to 20 April, and two snails survived until 14 May. When the last of the surviving snails was dissected on 17 May 1951, it contained 1839 developing and developed cercariae in the liver.

In our experiments, nine infected snails lived for over a year and all nine remained infected and shed cercariae until they died or were sectioned for examination. These observations lead to the conclusion that, during the larval development of *Paramphistomum microbothrium*, numerous successive generations of rediae maintain infection in the snail indefinitely. Only the death of the snail arrests this repeated production of new generations of cercariae of *P. microbothrium* in its intermediate host.

DEVELOPMENT IN A FINAL HOST

Encysted metacercariae obtained from experimentally infected snails were given by mouth to four cattle and four goats. In addition, a calf received encysted metacercariae obtained from naturally infected snails collected from the swamp at a cattle farm.

Post-mortem examination of the first steer was made on the 82nd day after it received 3000 cysts and 1665 young paramphistomes were recovered from the rumen. The length of these paramphistomes varied from 2·2 to 4·3 mm. At this stage of development all the male and female genital organs were already clearly recognizable, but the flukes had not reached sexual maturity. The ovary, Mehlis's gland, and testes were not fully developed, and the uterus contained neither spermatozoa nor eggs. In flukes of this size some morphological features characteristic of the species could already be discerned. The small testes were arranged in tandem one behind the other in the mid-line of the body. In the genital atrium,

which was located behind the junctions of the oesophagus with the gut caeca, the genital sphincter was well delimited from the surrounding tissues and the sphincter papillae were present. Laurer's canal was seen, its opening being situated just behind the excretory pore. The terminal ends of the gut caeca were turned to the dorsal surface of the body, where their ends were located between the excretory bladder and the dorsal wall of the body, posterior to the excretory and Laurer's canals.

The second steer was also given 3000 cysts and when it was autopsied 124 days later, 1757 young *P. microbothrium* were recovered from the rumen. The body length of the flukes varied from 2·5 to 7·1 mm. About half of these flukes had just reached maturity and the genital system had begun to function and produce sperms and eggs. In flukes whose body length exceeded 4·5 mm. the coiled vesicula seminales and pars musculosa were filled with spermatozoa, and the uterus contained from a few to a hundred eggs.

The third animal, the heifer, which received 3000 cysts, began passing eggs of *P. microbothrium* in the faeces 148 days after receiving the cysts. The number of eggs in the faeces increased steadily and reached during the 11th month of infection a maximum of 1640 eggs in 10 g. of faeces. Thereafter the number of eggs decreased suddenly and no eggs were found in the faeces after the 18th month of infection. Five months later this animal was autopsied and no flukes were found in its stomach.

The calf which was given about 2300 cysts obtained from naturally infested snails began passing fluke eggs in the faeces on the 98th day. The animal was slaughtered on the 147th day and 1125 specimens of *P. microbothrium* were recovered from the rumen. They were sexually mature although not fully grown. The conical body of these flukes varied from 6·0 to 8·0 mm. in length and from 1·8 to 2·1 mm. in breadth. The uterus contained several hundred eggs.

The number of cysts fed to one of the experimentally infected cattle could not be estimated, because the rubber tube containing 3000 cysts slipped out of the mouth of the animal, and a part of its contents was lost. This animal began passing eggs after 142 days. It was slaughtered on the 270th day and 121 specimens of *P. microbothrium* were found attached to the inner surface of its rumen. They were normally developed mature flukes varying from 8·5 to 10·5 mm. in length and from 2·7 to 3·0 mm. in breadth. The acetabulum measured from 1·9 to 2·2 mm. in diameter which represented a ratio of about 1:4·5 of the body length. The pharynx varied from 1·0 to 1·2 mm. in length. The deeply lobed testes, each comprising about fifteen lobes, were situated one behind the other in the middle third of the body length. Both were oval in outline in sagittal section and both were about 1·50 by 2·00 mm. in size. The genital pore was posterior to the level of the junction of the oesophagus with the gut caeca. The genital atrium was of the *Microbothrium* type. The ovary and Mehlis's gland were fully developed. The uterus, consisting of many coils, contained from 1000 to 2500 eggs per fluke, and spermatozoa. Laurer's canal ran along the excretory bladder, its opening being situated at the level of the excretory pore or slightly posterior to it. The terminal parts of the caeca, as is characteristic of the species, turned to the dorsal surface of the worm, where their

ends were situated one above the other between the excretory bladder and the dorsal wall of the body.

Miracidia were hatched from the eggs obtained from these specimens of *P. microbothrium* and were used successfully for experimental infection of snails (*Bulinus alluaudi*), in which the development of cercariae proceeded normally.

The four goats were given about 1000 encysted metacercariae each and slaughtered 147, 150, 181 and 214 days respectively after infection. The number of flukes recovered from these goats when it was compared with the large number of cysts given to them, was found to be surprisingly small. In the four animals only 146, 46, 150, and 157 specimens of *Paramphistomum microbothrium* respectively were found. In these goats, including the animal examined 214 days after receiving cysts, the flukes were very small. Their body length varied from 2·7 to 5·2 mm. Only half of the flukes contained eggs in the uterus which varied from eight to a 100 eggs per fluke. The goats apparently possessed some degree of natural resistance to *P. microbothrium*.

SUMMARY

The whole life cycle of *Paramphistomum microbothrium* Fischoeder, 1901, found in cattle of Kenya, East Africa, has been established experimentally.

In laboratory conditions, the eggs hatched miracidia on the 14th to 16th day, if they were kept in water at a temperature of 26–28° C.

In a snail, *Bulinus alluaudi* (Dautzenberg), kept at the temperature of 18–20° C., the miracidium developed into a sporocyst, the elongated body of which, containing young rediae, reached a length of 3·6 mm. in about 2 weeks.

The first rediae which emerged from a sporocyst were observed on the 14th day, and on the 20th day the first generation rediae began to produce second-generation rediae. From the 28th day onward the first-generation parent rediae ceased to produce daughter rediae and began to develop cercariae only. This period of production of cercariae by the redia lasted about 30 days, and when the life of the first-generation rediae drew to its close, the old rediae developed a few daughter rediae again.

Cercariae began to emerge from the first-generation rediae 30 days after exposure of the snail to miracidia. The cercariae left the parent redia in a very immature state and further development occurred in the liver of the snail. The emergence of cercariae from the infected snail began on the 43rd day after exposure to miracidia. Shortly after emerging from the snail, the cercariae attached themselves to vegetation and encysted.

Development of rediae of the second, third, fourth and apparently more successive generations followed an identical course to that outlined for the rediae of the first generation. As a result the successive generations of rediae maintain the infection going in an intermediate host and the infected snails were continually shedding cercariae as long as they lived. In the laboratory experiments the life span of some of the infected snails exceeded a year.

In cattle infected experimentally *P. microbothrium* reached maturity and began passing out the eggs about a 100 days after the encysted cercariae were fed to the animals.

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THE RELIABILITY OF THE CURRENT DIAGNOSTIC METHODS FOR THE IDENTIFICATION OF HELMINTH EGGS

By SHOOJI MORIYA

Department of Public Health, Osaka City Medical School, Asahi-cho, Abeno-ku, Osaka, Japan

(With 1 Figure in the Text)

INTRODUCTION

The diagnosis of the presence of helminths has mainly been done by detecting their eggs in the stools by concentration and other techniques. The reliability of these diagnostic techniques has not been fully studied with precision and I have undertaken a statistical study of this problem.

MATERIALS AND METHODS

The experimental material was 143 faecal specimens taken at random from 1316 individuals.

The unconcentrated faecal smears were made in the ordinary way, that is to say, a small representative fleck (about 0·05 g.) of the faecal specimen was thoroughly comminuted on a slide and covered with a 22 mm. square cover-slip. The amount of faeces taken was as constant (about 0·05 g.) as possible. The films were thin enough to enable a newspaper to be read through them.

The standard of diagnosis was the result obtained by the acid-ether centrifuga tion method with 3 g. of faeces.

The persons examining the films had had more than 3 years' experience of identification of helminth eggs in faeces, and were able to identify accurately the eggs of *Ascaris lumbricoides*, hook worms and *Trichuris trichiura*. In order to exclude errors due to personal variation in the individual workers, all the conditions were kept as constant as possible.

RESULTS

N faecal specimens (3 g. each) were separated into two groups, i.e. a positive group which included specimens containing helminth eggs and a negative group, which contained specimens free from them. If the ratio of these two were α and β respectively, $\alpha+\beta=1$, and each of the factors in the mother group are examined to determine the group to which they belong. The probability that the positive group will be diagnosed exactly as being positive is p, and it may be thought that the probability that the negative group will be diagnosed as negative is 1.

When the independent diagnosis has been done n times respectively with each of the composing factors of N materials, the probability that the α positive diagnosis is made k times repeatedly is P_k. Then

$$\left.\begin{aligned} P_k &= \alpha \frac{n}{k!(n-k)!} p^k (1-p)^{n-k}, \\ P_0 &= \alpha (1-p)^n + \beta = 1 - \alpha\{1-(1-p)^n\}, \end{aligned}\right\} \quad (0 < k \leqq n). \qquad (1)$$

If this diagnosis is done with all the faecal samples, and the number of the samples that are diagnosed as positive k times is M_k, then $\sum_{k=0}^{n} M_k = N$. And the likelihood (L) of obtaining such a result is as follows:

$$L = \frac{N!}{\prod_{k=0}^{n} M_k!} \prod_{k=0}^{n} P_k^{M_k}.$$

When this is substituted for (1),

$$L = \frac{N!}{\prod_{k=0}^{n} M_k!} \prod_{k=1}^{n} \left[\alpha^{M_k} \frac{(n!)^{M_k}}{(k)^{M_k} \{(n-k)!\}^{M_k}} p^{kM_k} (1-p)^{(n-k)M_k} \right] \left[1 - \alpha\{1-(1-p)^n\} \right]^{M_0}. \tag{2}$$

So that

$$\log L = C + \log \alpha \sum_{k=1}^{n} M_k + \log p \sum_{k=1}^{n} kM_k + \log(1-p) \sum_{k=1}^{n} (n-k) M_k + M_0 \log[1 - \alpha\{1-(1-p)^n\}]$$

(C is a constant, independent of α and β.)

Therefore, $\hat{\alpha}$ and $\hat{p}$, which are the Maximum Likelihood Estimate of α and p respectively, are given by solving the following simultaneous equation

$$\left.\begin{aligned} \left.\frac{\partial \log L}{\partial \alpha}\right|_{\alpha=\hat{\alpha},\, p=\hat{p}} &= \frac{\sum_{k=1}^{n} M_k}{\hat{\alpha}} - \frac{M_0\{1-(1-\hat{p})^n\}}{1-\hat{\alpha}\{1-(1-\hat{p})^n\}} = 0, \\ \left.\frac{\partial \log}{\partial p}\right|_{\alpha=\hat{\alpha},\, p=\hat{p}} &= \frac{\sum_{k=1}^{n} kM_k}{\hat{p}} - \frac{\sum_{k=1}^{n} (n-k) M_k}{1-\hat{p}} - \frac{n(1-\hat{p})^{n-1}\hat{\alpha} M_0}{1-\hat{\alpha}\{1-(1-\hat{p})^n\}} = 0. \end{aligned}\right\} \tag{3}$$

When this equation is simplified,

$$\hat{\alpha} = \frac{N - M_0}{N\{1-(1-\hat{p})^n\}}, \tag{A}$$

$$\sum_{k=1}^{n} M_k k - Nn\hat{p}\hat{\alpha} = 0. \tag{B}$$

If $n > 4$, we cannot solve this equation algebraically, so that, to begin with, we substitute (A) for (B), and we then get the following

$$\sum_{k=1}^{n} kM_k - (1-\hat{p})^n \sum_{k=1}^{n} kM_k - n\hat{p}(N - M_0) = 0. \tag{4}$$

The approximate value of p can be obtained easily by Newton's method, because $0 < \hat{p} \leqq 1$,

$$\left.\begin{aligned} \hat{p} &= \frac{\sum_{k=1}^{n} kM_k}{Nn\hat{\alpha}}, \\ \alpha &= \frac{\Sigma kM_k}{\hat{p}} \end{aligned}\right\} \tag{5}$$

Table 1. *The result obtained from the examinations of four smears per single faecal specimen*

No. of times, diagnosed as positive ...	0	I	II	III	IV
Ascaris lumbricoides	83	4	7	14	35
Hook worm	103	18	9	7	6
Trichuris trichiura	86	29	19	5	4

Accordingly we can get $\hat{\alpha}$ and $\hat{p}$ from the simultaneous equations (4) and (5).

When four smears of each specimen were examined and the results (shown in Table 1) were applied to the simultaneous equations given above, the values of $\hat{\alpha}$ and $\hat{p}$, shown in Table 2, were obtained.

Table 2. *The values of $\hat{\alpha}$ and $\hat{p}$*

	$\hat{\alpha}$	$\hat{p}$
Ascaris lumbricoides	0·4197	0·833
Hook worm	0·3045	0·465
Trichuris trichiura	0·5099	0·336

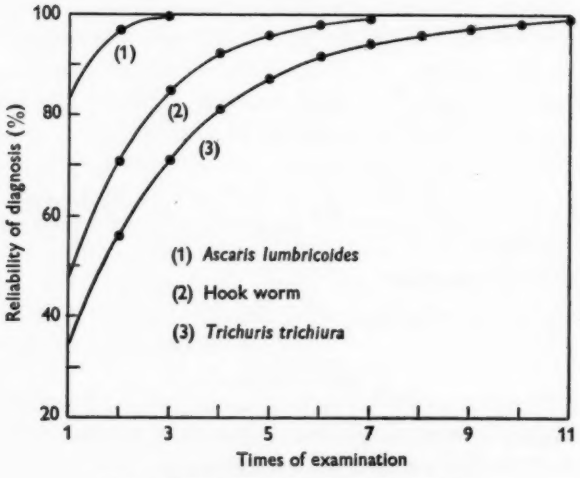

Fig. 1. The diagram of the values of P_k.

The probabilities of diagnosing positive preparations positive by one inspection were thus 83·3 % for *Ascaris lumbricoides*, 46·5 % for hook worm and 33·6 % for *Trichuris trichiura*.

When the number of examinations of each smear is increased, the probability P_k may be obtained by the following equation:

$$P_k = 1-(1-p)^k.$$

Table 3 and Fig. 1 show the results then obtained.

Table 3. *The values of* P_k

Times of examination ...	1	2	3	4	5	6
Ascaris lumbricoides	0·833	0·972	*0·995*	0·999	—	—
Hook worm	0·465	0·713	0·846	0·918	0·956	0·976
Trichuris trichiura	0·336	0·559	0·708	0·806	0·871	0·915
Times of examination ...	7	8	9	10	11	12
Ascaris lumbricoides	—	—	—	—	—	—
Hook worm	*0·987*	0·993	0·996	0·998	—	—
Trichuris trichiura	0·944	0·963	0·975	0·984	*0·989*	0·993

DISCUSSION

Assuming that the limit of the signification is 1 %, to exceed the value of p over 99 % we must make three examinations for *Ascaris lumbricoides*, seven for hook worm and eleven for *Trichuris trichiura*. That is to say, for the diagnosis of the presence of the eggs of these three species of worms, it is necessary to make these numbers of examinations by the direct smear method in order to obtain the result obtained by the concentration method using 3 g. of faeces. The figures given above represent quite fairly our experience of the identification of the helminth eggs in stools.

SUMMARY

1. On the assumption that the limit of significant difference is 1 %, it is necessary, when concentration methods are not used, to repeat the faecal examination 3 times for the egg of *Ascaris lumbricoides*, 7 times for hook worm and 11 times for *Trichuris trichiura* in order to obtain the diagnostic results obtained by the acid-ether concentration technique using 3 g. of faeces.

2. The coefficient of the identification by a single smear examination is 83·3 % for eggs of *Ascaris lumbricoides*, 46·5 % for hook-worm eggs and 33·6 % for eggs of *Trichuris trichiura*.

The author wishes to express his thanks to Dr H. Sugiyama for his valuable suggestions and to Mr J. Fukushima, M. Fukuda and I. Isokawa for their kind help.

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BREEDING THE INDIAN HOUSE-FLY (*MUSCA DOMESTICA NEBULO* FABR.) FOR EXPERIMENTAL STUDIES

By P. J. DEORAS

Haffkine Institute, Bombay 12*

(With 5 Figures in the Text)

INTRODUCTION

Grady (1928) and Peet (1932) introduced methods for breeding flies throughout the year. Hockenyos (1931), Richardson (1932), David & Harvey (1941), Roy & Siddons (1940) and Basden (1947) have tried to improve upon the method of breeding flies for experimental purposes. Some of these methods are complicated, and flies of a definite race, and age were not available for morphological, physiological, genetical and disease transmission studies as and when desired under tropical conditions.

Attempts were therefore made in 1946 to breed the Indian house-fly, and a short note on that subject was published (Deoras, 1948). The breeding was done under artificial lighting conditions. For the last five years breeding has been done under natural lighting conditions, and the method now described seems adaptable to tropical conditions for the studies above noted.

TECHNIQUE

Equipment

(1) *Laboratory*

The insect was first bred in Bombay in a small room in a basement where the only window was covered by an exhaust fan and the breeding was done under artificial lighting for all the 24 hr. The average temperature for the 14 months was 30° C. and the average relative humidity 82%. It was found that the flies emerged quicker from pupae under these conditions than in the normal course.

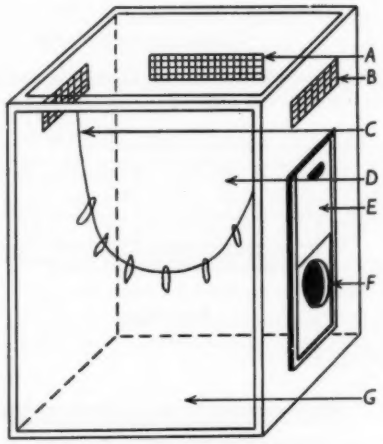
The breeding was therefore started in a newly built room 14 ft. long and 8 ft. broad and 8 ft. high. One of the long walls faces north and is made entirely of ground glass with embedded wire gauze. This gave ample daylight for work. There is a double door on one side and a special window on the other. The window consists of an opening 3 ft. high and $1\frac{1}{2}$ ft. wide. This window is covered by a slanting ground glass from outside in such a manner that an opening $1\frac{1}{2} \times 1\frac{1}{2}$ ft. is left at the base of the outside of the window. This opening is covered by 24-mesh copper wire gauze. The sides are covered by glass. The ventilation in the room is maintained by a small exhaust fan on the door side of the wall, which, while working, draws a gentle breeze from the wire-gauze base of the window. A rough variation in the temperature prevailing in the room is brought about either by putting a wet cloth

* This work was started and is continued at the Research Institute of the Maharashtra Association for the Cultivation of Science, Poona 4.

over the wire gauze to give a cool breeze or putting a gas flame for a hotter temperature. The average temperature of the room has been 30° C. and the average humidity 65 %. A table, with a sink, inside the glass wall enables two workers to do the studies comfortably, and the cages are stacked on iron racks on the opposite side. The room is situated away from other laboratories and is surrounded by a small garden. No insecticide is brought near the room. Genetical and physiological studies were done inside the room.

(2) *Cages*

(*a*) *Rearing cage* (Fig. 1). This is a wooden structure 41 cm. high, 17 cm. wide and 35·5 cm. long. The front is made up of a complete sliding door (*D*). Three sides of the cage bear slits covered by a fine wire gauze (*A*, *B*). One side of the cage has a circular opening of about 8 cm. in diameter (*F*) closed by a small sliding door (*E*). A string (*C*) is suspended from the cage top for the flies to rest upon and sip the food put there on cotton wads. A Petri dish containing larval food for oviposition is kept at the bottom of the cage (*G*).



Rearing cage (for adult)

Fig. 1. Rearing cage. *A*, *B*, aeration openings; *C*, string for suspending the fly food; *D*, sliding glass door forming the front side of the cage; *E*, covering door to the opening; *F*, opening to the cage; *G*, bottom of the cage.

(*b*) *Breeding cage* (Fig. 2). This rectangular cage is 27 cm. long, 15 cm. high and 21·5 cm. wide. It is made of galvanized sheet iron with a covering of 16-mesh wire gauze (*B*). A central strip of sheet metal (*A*) bears hooks inside from which adult fly food may be suspended. One side of this cage (*D*) has a circular opening, 9·5 cm. in diameter, which is covered by a tin cap (*E*). At the bottom of the cage lies a 0·5 cm. layer of loose dry earth on which are kept the Petri dishes brought from the rearing cages or the stock cage after oviposition.

(*c*) *Stock cage* (Fig. 3). This galvanized sheet iron cage is 46 cm. long, 36 cm. high and 27 cm. wide. The roof of the cage and two sides have an opening (*A*, *D*),

9 cm. in diameter. The cover for the roof opening is fitted with a plug for an electric light bulb. The sides of the cage at the upper levels bear slits (*C*) covered with fine wire gauze. These slits transmit natural light to the cage. Two hollow perforated iron pipes 3 cm. in diameter (*B*) run from the bottom of the cage and bulge at the top end above the roof. These are for aeration. The entire cage rests on legs and is

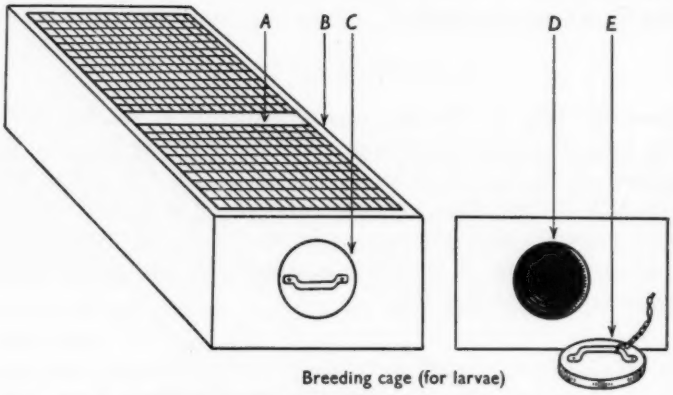


Fig. 2. Breeding cage. *A*, central tin strip; *B*, wire gauze cover to the cage; *C–D*, opening to the cage; *E*, cover to this opening.

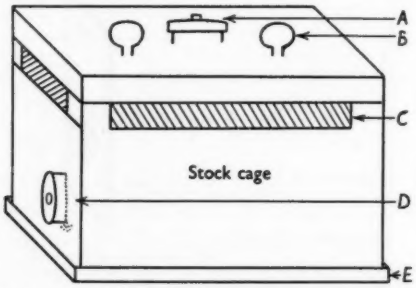


Fig. 3. Stock cage. *A*, the upper opening with an electric plug and holder; *B*, aeration pipes; *C*, aeration slits; *D*, side opening; *E*, tray.

contained in a tray (*E*) which has a little water in it. Criss-cross strings are suspended from the top of the cage to serve as resting places for flies and also for supporting the fly food on cotton wads. Cultures of flies in this cage give a regular supply of eggs. More eggs were obtained with a continuous blue light in the cage than with red, yellow or normal electric light.

(3) *Food*

(*a*) *Adult*: (1) milk, 5 g.; (2) cane sugar 10–15 g.; (3) banana (fresh), 25 g. (about); (4) agar agar, 1 g.; (5) formalin, 2 drops; (6) water, 250 ml.

Fresh ripe banana is crushed in a mortar to a fine paste, and the sugar is ground in. The milk powder is gradually added, followed by the agar agar which had been

soaked in water overnight. After adding the formalin, the mixture is made up by the addition of the requisite quantity of water. Cotton wads dipped in this mixture are spread over the strings in the rearing and stock cages for the flies to sip and feed. They alight on the suspended strings as is their normal habit. These wads of food are changed each day. Slight variation in the procedure noted above curdled the milk which became unsuitable for the flies. This food, if kept in Petri dishes, not only makes the wings sticky but also drowns the flies.

(*b*) *Larval*: (1) wheat bran, 10 parts; (2) Quaker oats, 1 part; (3) yeast water (4 tablets of 4 grain each in 200 ml. of water), 10 ml.; (4) water, about 50 ml. to give proper consistency to the soft dough.

The wheat bran and oats were mixed and the yeast solution gradually added. Sufficient water was then added to give the material the required consistency which is suitable for oviposition. This food, in Petri dishes, is kept in the rearing and stock cages for egg laying. It is removed the next day (when eggs have already been laid) and kept in the breeding cages. If no eggs are laid the food is discarded.

(4) *Method of working*

Eggs. These were laid in bunches in the crevices of the moist food. They hatch within 6–12 hr. and the larvae burrow in the $\frac{1}{2}$ in. layer of the food. Dishes in which eggs were laid on the same day were kept in one breeding cage. This was convenient for obtaining flies of known age.

Larvae. Fresh food was added each day over the dried crust of the larval food of the previous day. As the food dries up, the larvae have a tendency to leave the Petri dish. It takes about 4 days for the larvae to grow and start migrating to the soil kept below the Petri dish. The addition of food to the dish is then stopped and no attention is paid to the cage until the first flies emerge. If the larvae were too crowded an extra food dish was put in and some of the larvae transferred to it. Otherwise, overcrowding may cause the larvae to migrate to the soil and pupate prematurely or die. Such premature pupae either died or gave rise to very small and weak flies.

Pupae. Flies emerged in such cages 4 days after pupation. The cage was discontinued after the 10th day. Before discarding the cage the number of empty puparia, the number of unbroken puparia, and the number of dead flies in the cage were counted to give an idea of the percentage of emergence and the early mortality in these flies. The early pupae in the cage if kept in a frigidaire at 4° C. survived for 20 days and then emerged. There was not much effect of this temperature on the late pupae.

The soil in the cage was sifted and made sterile by heating before using it again in another cage.

Imago. All flies emerging in various breeding cages on the same day were transferred to one or two rearing cages according to the number present. The flies feed, mate and start oviposition after the 3rd day. Thus the 4-day-old fly was taken to be mature for biological tests of insecticides.

To transfer flies from the breeding to rearing cages, the openings were brought close together, the wire gauze of the breeding cage was covered by a black cloth,

and the flies flew towards the light coming from the glass end of the rearing cage. To collect adult flies for experiments, a hand was inserted with a test-tube in the rearing cage and the glass side directed towards the light. All the flies flew towards the glass and they were collected by an upward sweep of the open end of the test-tube. The tube was closed by the thumb, taken out, and plugged with cotton prior to dispatch for experiments. Flies not needed for experiments after the 4th day are kept in stock cage for further egg-laying.

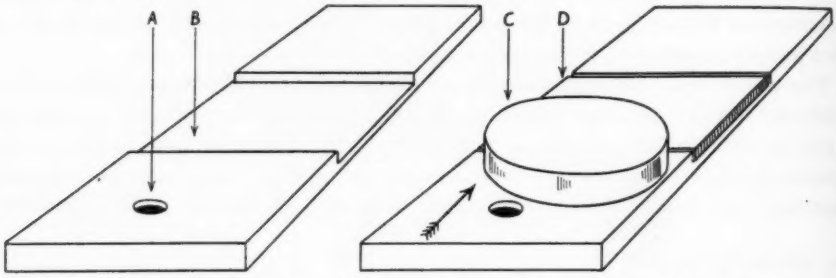


Fig. 4. Gadget for testing flies. *A*, half inch hole; *B*, scooped area of the wood block; *C*, Petri dish sliding over to the glass slab; *D*, glass slab.

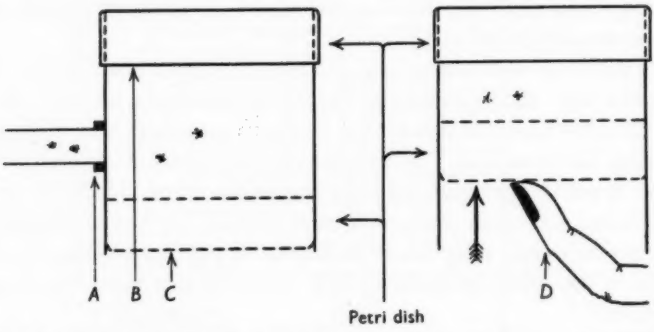


Fig. 5. Gadget for examining flies. *A*, test-tube over the tin entrance; *B*, upper Petri dish; *C*, lower Petri dish; *D*, finger pushing the lower Petri dish upwards.

(5) *Gadgets*

(*a*) *To transfer flies from a test-tube to a slab of glass* (Fig. 4). This gadget consists of a rectangular block of wood, the central part of the upper surface of which is cut away to a depth equal to the thickness of the slab of glass (*B*). The glass plate lies in this hollow. The flies are introduced from a test-tube at the bottom of a small opening (*A*) over which lies a Petri dish. The Petri dish (*C*) covering the flies is slowly slid across to the glass plate. This gadget is useful when flies are needed for testing any liquid or solid lying on the glass slab. The plate, with the flies beneath the Petri dish cover, could be moved to a binocular for a close observation of the reactions of the flies. The reverse of this method could be used to guide flies into thin tubes for close observations.

(*b*) *To transfer flies from a test-tube to between two Petri dishes* (Fig. 5). A short

cylinder of thin tin with an internal diameter just sufficient to hold the lower half of a Petri dish (*C*) and over which the top half of the Petri dish would fit as a lid (*B*), is prepared with a ½ in. hole on one side (*A*). Flies in a test-tube are introduced through this hole into the ring. The lower Petri dish is slowly pushed up till its rim touches the upper dish, and both are then removed with flies enclosed between them. This gadget was found useful for testing any dust in the dish or for observing at close range the habits of flies.

DISCUSSION

The advantages of the method of breeding described above are that flies of a pure race and known age are obtainable at any time. Secondly, the breeding technique does not involve any complicated process whereby the eggs, larvae or pupae have to be touched and disturbed which may result in getting deformed flies. Thirdly, the steps in rearing are so few and simple that with little attention an unskilled worker can look after the breeding under tropical conditions. Fourthly, the running expenses for maintaining the colony by a part-time worker come to less than Rs. 25 per month, depending upon the requirements. The only trouble that is often encountered is the growth of fungus on the larval food and the parasitization of the pupae by chalcids. The former is avoided by rinsing the apparatus every fortnight with 1/1000 solution of mercuric chloride and the latter by always keeping the room closed.

SUMMARY

1. A technique is described for the laboratory breeding of *Musca domestica nebulo* Fabr. the Indian house-fly.

2. Methods are described for the transferring of the flies from cages to appliances for testing insecticides and for close observation.

I am very thankful to Mr E. B. Raj-der-ker who kindly donated the B. K. Raj-der-ker insect breeding station for our work. I owe a debt of gratitude to the authorities of the Indian Law Society, Poona, who have placed land and building facilities at our disposal for this research. Prof. S. P. Agharkar placed all the facilities of the Research Institute of the Maharashtra Association for the Cultivation of Science at my disposal, and Mr D. R. Ranade voluntarily supervised the breeding at Poona.

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ON THE STRUCTURE OF THE SPIRACLE OF THE TICK, *ORNITHODOROS MOUBATA* MURRAY

By T. O. BROWNING*

Department of Zoology, University of Cambridge

(With 1 Figure in the Text)

The most conspicuous feature of the spiracle of *Ornithodoros moubata* is the so-called cribriform plate. This is a massive crescent-shaped vesicle consisting of three layers; the underlying sclerotized endocuticle, a series of branching pillars or pedicels arising from this basal layer and a very thin outer membrane, supported on the distal ends of the pillars and enclosing the whole structure (Fig. 1A). The cavity lying between the basal cuticle and the outer membrane and ramifying between the pillars is continuous with the atrium of the spiracle.

A tongue of hard cuticle, the macula, projects into the inner curve of the crescent enclosing a slit-like aperture, the ostium, connecting the atrium with the exterior.

Both genera of the Argasidae have spiracles of this type (Robinson & Davidson, 1913; Cunliffe, 1921; Mellanby, 1935). In the Ixodidae the spiracles are much larger but the structure is essentially similar. However, in this case the ostium and macula are situated in the centre of a circular or pear-shaped plate (Nuttall, Cooper & Robinson, 1908).

In 1891 Batelli, making what was probably the first detailed study of the spiracle of a tick, described the spiracular plate of *Ixodes hexagonus*. Here the pillars anastomose at their distal ends forming an irregular hexagonal 'meshwork' which supports the thin covering membrane. He stated that the membrane was pierced in the centre of each hexagon by a small pore which served to connect the air in the spaces between the pillars with the external air. In 1908, Nuttall, Cooper & Robinson, making a very detailed study of the spiracle of *Haemaphysalis punctata*, also concluded that the external membrane was perforated by numerous small pores. Robinson & Davidson (1913) described the outer membrane of the spiracle of *Argas persicus* as porous. Mellanby (1935) gave a description of the spiracle of *Ornithodoros moubata*, showing the outer membrane of the plate, in a rather diagrammatic figure, as being perforate.

In nature many ticks live in very arid environments, and in the laboratory are known to be able to survive for long periods in dry air. Such capacity to withstand desiccation seems unlikely if the tracheal system were indeed permanently open to the air, for in many insects it is known that causing the spiracles to remain permanently open results in a marked increase in the rate at which water is evaporated in dry air (Wigglesworth, 1950*a*). It therefore seemed worth while to

* Nuffield Foundation Dominion Travelling Fellow in natural science. On leave from the Waite Agricultural Research Institute, University of Adelaide.

examine the spiracles of a tick to see if the outer covering of the spiracular plate were indeed porous.

Sections were cut of the spiracle of *O. moubata* and whole mounts of spiracles both from fresh ticks and from exuviae were examined. As shown in Fig. 1A the outer membrane is exceedingly thin in places, probably consisting of epicuticle alone at these points, but in no case was a definite porous structure observed. The tracheal system was filled with cobalt sulphide by the method described by Wigglesworth (1950*b*) and sections were cut and examined, but there was no suggestion of the black sulphide penetrating the outer membrane, as would be the case if pores existed in it. It was concluded that in *O. moubata*, at least, the outer surface of the spiracular plate is continuous.

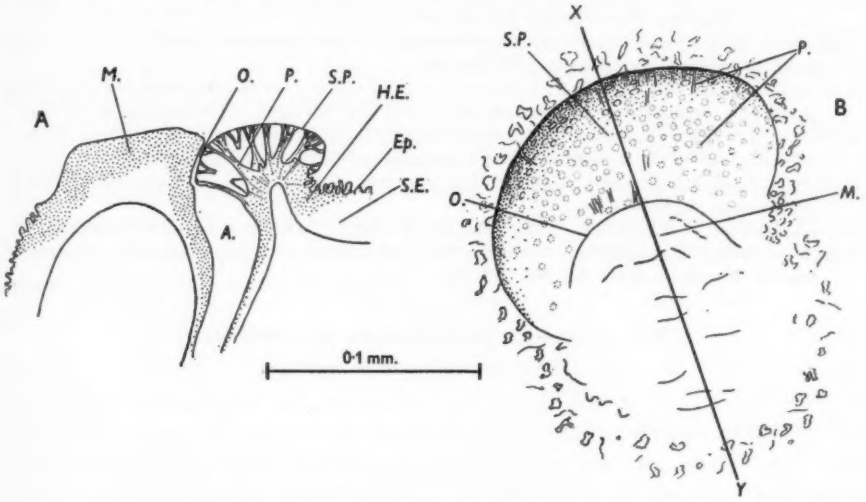

Fig. 1. A: transverse section of the spiracle of *O. moubata* about in the plane *XY* in B. B: the spiracle in surface view. The spiracle is oriented with its dorsal aspect towards the top of the figure and its anterior towards the left (cf. Mellanby, 1935). *A.*, atrium; *Ep.*, epicuticle; *H.E.*, sclerotized endocuticle; *M.*, macula; *O.*, ostium; *P.*, pillar or pedicel; *S.E.*, soft endocuticle; *S.P.*, spiracular plate.

In surface view the spiracular plate gives the appearance of being porous (Fig. 1B). On examination the apparent pores are seen to be the expanded distal ends of the pillars where they join the surface membrane. In Ixodidae this illusion does not occur and the impression of holes in the outer membrane is probably due to the dermal glands, which open between the bases of the pillars, being visible in whole mounts through the transparent outer membrane (Nuttall, Cooper & Robinson, 1908).

The terms 'cribriform plate' (Nuttall, Warburton, Cooper & Robinson, 1908; Cunliffe, 1921; Mellanby, 1935) and 'area porosa' (Robinson & Davidson, 1913) which have been used to describe this structure in ticks are thus misleading. The term 'spiracular plate' would perhaps be a better name for this very characteristic feature of the spiracles of ticks.

The function of the spiracular plate, in Argasidae at least, seems to be to provide a pad against which the macula can impinge when it is depressed. Because of the flexibility inherent in its structure and the relative hardness and inflexibility of the macula the result should be a very efficient seal of the ostium, a necessary condition for animals so likely to be exposed to desiccation.

Grateful acknowledgement is made to Prof. V. B. Wigglesworth, F.R.S., in whose laboratory this work was done.

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THE ECOLOGY OF THE IMMATURE PHASES OF TRICHOSTRONGYLE PARASITES

V. THE ESTIMATION OF PASTURE INFESTATION

By H. D. CROFTON, B.Sc., Ph.D.
Department of Zoology, University of Bristol

(With 3 Figures in the Text)

INTRODUCTION

A considerable contribution to the study of parasitic populations was made when Taylor (1939) described a method of sampling pastures to determine the number of infective larvae available to grazing hosts. This method, although it has many drawbacks, has never been superseded.

During the last twelve years the writer has used Taylor's method and tried a number of modifications of it. Some of these are described below. While attempting to relate pasture infestation with infections occurring in the sheep grazing them it has been realized that the interpretation of results of sampling is as important as the choice of technique and work is described to relate techniques to problems of parasite populations on sheep pastures.

I. METHODS

(*a*) *Methods of sampling*

Essentially all methods are dependent upon, and limited by, the fact that the volume of herbage is enormously large in relation to the volume of larvae. The obvious approach in such circumstances is to use a sampling method and three main techniques used are described and discussed below. The first two involve grass sampling methods, while the third is a method of trapping larvae.

The significance of the results from these methods cannot be discussed until other questions have been considered, but it is appropriate to state here that the original method was based upon an assumption that the larvae are randomly distributed on pastures. It has been shown in a previous paper (Crofton, 1952) that this is not a valid assumption.

(i) *Taylor's method*

Basically this involves a system of traversing a pasture and taking samples at measured intervals. It is adequately described by Taylor (1939). Strictly, samples are not taken at random and randomness is sacrificed to prevent local choice during the traverse. This may be justified on general grounds, as will be seen later, and it is probable that with pooling of samples as suggested by Taylor, the departure from randomness is not very important. The question as to the advisability of pooling the samples, with consequent loss of information must be considered as part of the general problem of estimation, but the reason for it is the difficulty of handling very large numbers of small samples by the method of recovery described.

The choice of paths of traverse of the pasture is obviously important, and in theory the number of traverses suggested by Taylor is much too low to ensure efficient sampling. Quite obviously a compromise must be made between a theoretically ideal and a practical method. If the number of traverses were increased so each acre were crossed six times and a single 'pinch' taken every 10 yards then the number of pinches would only be about fifty per acre or about one pinch per 100 sq. yd. If the larvae were very numerous and randomly distributed then such a rate of sampling might be sufficient, but because the larvae are neither randomly distributed nor numerous in relation to the total number of pinches an acre of pasture could provide, it seems obvious that the number of samples must be either greatly increased in number or in size. In either case the increased bulk of herbage makes recovery techniques more difficult.

(ii) *Grass cutting*

Leaving for the time the number and size of samples to be taken, it is obvious that the method of taking samples can be of importance. In Taylor's method the samples are taken by pulling small 'bunches' of grass using the forefinger and thumb. This, to some extent, simulates the cropping of grass by sheep, but, unless great care is taken very unrepresentative samples are obtained because soil and 'mat' are removed with the grass blades. It has been shown (Crofton, 1948) for *Trichostrongylus retortaeformis* that the greatest density of larvae occur in the 'mat', so that sampling for larvae can be greatly affected by the distance at which the sample is taken from the soil surface; uprooting of grass will tend to give higher counts than plucking above the surface of the mat. Methods of grass cutting have been tested, using both scissors and a small rotating blade and collecting box. In the case of the scissors method it is not easy to decide upon and maintain a fixed level. The process is exceedingly laborious and this tends to reduce the number of samples which can be taken. In the case of the rotating blade and box the level of cutting can, in theory, be predetermined and maintained, but this only applies where the ground is smooth and even and where the grass is not too long. These latter conditions seem to be rarely satisfied and there seems to be little or no advantage in using the mechanical type of cutter except in so far as sampling involves less effort and a large number of samples can be taken fairly quickly. One defect of the mechanical cutter is that even after careful adjustment there is a tendency for sampling to be selective.

Perhaps the most important factor to consider in comparing 'plucking' with cutting is the different levels from which samples may be taken. It is possible to remove grass by cutting at the base of the blades, although it is difficult to do this consistently. 'Plucking' usually gives the whole of the grass blade and some 'mat' and soil. It has been mentioned previously that the majority of larvae are found on the lower parts of the blades and in the 'mat', and any estimate of the total population of larvae must be concerned with the 'mat' and possibly with the soil as well as with the grass blades. It may be argued that the estimate required is of the larvae available to the grazing animal, and that the larvae available to sheep might be considered (arbitrarily) to be those above the 'mat' because under normal

conditions of grazing the mat and soil are not disturbed. Such an estimate could be obtained by using a grass-cutting method (either scissors or mechanical cutter), but it is to be noted that slight alterations in level of cutting may result in great variations in the number of larvae sample (see Table 1). Not only will this make it

Table 1. *The distribution of larvae along grass blades*

(Data obtained from experimental plots on four occasions previously inoculated with 5000 mixed trichostrongyles (mainly Ostertagia).)

	No. of larvae			
Sample taken	Plot 1	Plot 2	Plot 3	Plot 4
At soil level	920	1110	740	1420
½ in. above soil	1450	890	1250	910
1 in. above soil	790	650	420	130
1½ in. above soil	120	40	120	40
2 in. above soil	20	10	40	4

difficult to compare results of one worker with that of another, but it will also make difficult the comparisons of samples taken by the same worker from different fields. A more fundamental difficulty arises, however, if samples are considered only in terms of 'available larvae'. This tends to imply a static population such that a sample at any height is representative of the total population. The proportion of larvae at different heights in the herbage varies considerably according to changes in the micro-climate (see Crofton, 1948), and the larval population on grass blades can never be considered to be static because the number of 'available' larvae is a very complex function of the number of larvae on the soil and 'mat'. Even though there is no exchange of larvae from blades to 'mat', there must be transfer from 'mat' to blades.

Plucking, while not completely satisfactory, is likely to give more consistent results because it involves sampling a greater part of the larval population, and the results will be less dependent upon weather conditions. In practice the amount of soil removed varies according to the pasture, its structure and past history, and to the person taking the sample. Such variations in soil removal may have a considerable effect on the estimates, but over a large number of samples this source of variation is less important than that produced by a cutting method where only the fringe of the distribution is sampled. As will be seen later, however, the presence of soil in samples makes recovery technique more difficult and probably less reliable.

(iii) *Trapping*

In any trapping method the movement of the animals to be trapped is used to produce concentrations of populations derived from an area or volume much greater than that occupied by the trap. Usually the animals moving into the trap either do so randomly or as a result of some 'attractant' which may vary from a source of light to specific chemicals. Once in the trap the animal is captured either mechanically or by the exploitation of some intrinsic behaviour pattern. The area

or volume sampled by a single trap is directly related to the rate of movement of the animals and also to the time for which the trap is in position.

Methods of trapping infective larvae of trichostrongyles are limited because there is little lateral movement (see Augustine, 1923; Monnig, 1930; Furman, 1944; Dinaburg, 1944) so that the area sampled must be the same or approximately the same as the area of the trap, and the main object of trapping is not achieved. Given suitable conditions in the trap, however, it might be expected that larvae could be efficiently separated from the herbage in the area of the trap. 'Suitable conditions' means in this case, conditions suitable for vertical migration. Thus, at best, a trapping method could be a highly efficient method for separation of larvae from small areas of herbage. For sampling large areas, such as a field, the number of traps would have to be great or the size of each trap exceedingly large. In fact, trapping, in the true sense, is only possible in the vertical plane and the use of large numbers of traps in a field is equivalent to taking a large number of samples equal in number and area to the number of traps and area occupied by the traps. Thus the so-called trapping method is only a method of separation of larvae from the herbage.

A series of traps were made, using heavy glass dishes 10 cm. in diameter and 5 cm. deep. They were placed open end downwards on a pasture and fastened firmly to the ground by wire clips. They were left in position for 2 days and then the inner surface was washed and the washings examined for larvae. Using twenty traps on two occasions only one trap was found to contain larvae. Later work has shown that when using only forty traps over an unselected area, one positive result is higher than the normal expectation. To check the efficiency of the traps they were placed over freshly passed faeces and left for 8 days. Large numbers of larvae were recovered from all the traps.

(*b*) *Separation of larvae*

As has been indicated, most methods of sampling involve the separation of larvae from relatively large amounts of grass and soil. In all methods it is necessary to differentiate between stages of free-living nematodes, and infective larvae.

(i) *Taylor's method*

This is described by Taylor (1939) and involves grass washing and sedimentation, which is followed by concentration on filter-paper and subsequent separation of the larvae from the sediment in a Baerman funnel. The inefficiency of the Baerman funnel separation and the loss of larvae during sedimentation was recognized by Taylor. He recommended the use of an additional series of samples to which have been added a known number of larvae to estimate the proportional loss during the various processes. It is obvious that this part of Taylor's technique can be used with advantage in any method adopted. The original method is laborious, and although various modifications can be made, e.g. the use of funnels for washing and sedimentation, it is difficult to use on a large scale.

(ii) *Flotation method*

In this method, which has been used with some success, the grass sample is moistened after weighing so that the grass is completely wet, but without excess

water. To this is added a flotation medium (sat. zinc sulphate) in small quantities until the grass is covered. After 5 min. the grass is then covered by a wide mesh grid to prevent individual blades rising to the surface. (In practice it is found that a $\frac{1}{4}$ in. mesh grid is sufficiently fine—the small amount of grass escaping being easily separated by hand.) Flotation medium is added until a hydrometer shows that an 85 % saturated solution of zinc sulphate is present. The flotation medium is allowed to stand for 5 min. and then decanted into a vessel shown in Fig. 1. In this container the larvae rise to the top and may be flushed out with a small additional amount of $ZnSO_4$. The overflow is caught in a small jar, and the liquid examined for larvae which are then counted.

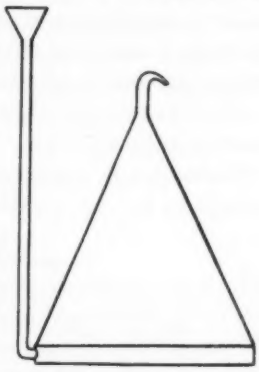

Fig. 1. Apparatus used in flotation method of separating larvae from herbage.

(iii) *Identification of larvae*

The separation of infective larvae from those of free-living forms is, in theory, easy. It involves the recognition of larvae of well-known and well-described species (see Dikmans & Andrews, 1933, Andrews, 1935). Unfortunately several larvae of free-living species found on pasture and in soil are superficially similar to trichostrongyle larvae, and while careful examination and comparison will separate the infective larvae from others, this cannot be done speedily, especially, as sometimes happens, when free-living nematodes are very numerous. Taylor (1939) attempted to simplify the task, and to free the larvae from debris, by allowing the mixture of larvae to dry slowly on filter-paper. He depended upon the fact that while infective larvae of trichostrongyles recover after being dried in this way, most free-living forms do not. This method is useful, but some free-living forms survive. The use of $ZnSO_4$ solution dehydrates the larvae, giving an effect similar to drying. If the strength of the flotation medium is increased slowly the infective larvae curl in a tight spiral, usually leaving only the tail clearly exposed. Most of the free-living forms remain straight or slightly curved. Both of these methods reduce the number of larvae to be examined carefully. The addition of $ZnSO_4$, or if Taylor's original method is used, the addition of saturated salt solution to the extracted larvae, is more rapid, easier and usually more reliable than the drying method, because in this latter case the exact stage of drying is sometimes difficult to judge. If necessary the larvae treated with $ZnSO_4$ or saturated salt solution can be restored to activity by the addition of sufficient water.

II. THE DISTRIBUTION OF LARVAE ON PASTURES

An important factor which must be considered in relation to methods of sampling is the form of distribution of larvae on pastures. In a previous paper (Crofton, 1952) it has been shown that the larvae are not distributed randomly, and that the distribution approximates to a form described by Neymann as a contagious distribution. This type of distribution might be expected when it is realized that the larvae develop in clumps of faeces scattered over the pasture.

(a) Contagious distribution

In the work described previously (Crofton, 1952) samples were taken from a selected portion of pasture which, from a survey of faecal pellets, appeared to have been evenly grazed. The result of this selection of the pasture was to give a bias to the results, but this was intentional in order that the simplest case might be considered. It is to be noted that even in this simple case comparison of results from different fields is difficult, and that while a logarithmic transformation might enable a comparison to be made, the validity of such a transformation is doubtful. Perhaps a more justifiable approach would be to compare the parameters of the Neymann distribution from results obtained by sampling different fields.

Such a comparison has been made by Beall (1940) using data from observations on *Pyraustra nubilalis*.

Using Beall's notation, where the parameters of Neyman's type A are represented by m_1 and m_2, then,

$$m_1 = \alpha t, \qquad m_2 = \beta/t \text{ (after Beall)};$$

where α = number of clumps of faeces per unit area, β = average number of larvae surviving from each clump, t = area of clump of faeces from which larvae develop. Neyman's distribution assumes that the larvae are all at the same state of development, and this will in fact be true within the limits that only third-stage larvae will have migrated from the clumps of faeces. We then require to estimate the number of larvae occurring in a unit area W, these larvae being derived from an area, t. Because of the very limited amount of lateral movement of larvae, $w = t$; and in comparisons between fields a and b it may be assumed that $t_a = t_b$. The difference between the fields can then be determined by comparing values of α and β and this can be done by comparing the parameters m_1 and m_2 for the distributions on each field. In practice α cannot be estimated because of the high rate of breakdown of clumps of faeces, and the difficulty of distinguishing between recently passed faeces and faeces from which the larvae may have migrated. However, with a knowledge of the previous stocking of the field it is possible to estimate the relative values of α_1 and α_2 for the fields a and b under comparison, and α_1 and α_2 will, if the fields have been stocked with sheep for the same length of time, be proportional to the stocking rates, i.e. the number of sheep per unit area. Even a difference of stocking time will not greatly affect the estimates in summer because of the rapid hatching and disappearance of larvae. Thus a comparison of m_{a1} and m_{b1} will give an estimate of difference in rate of stocking and faeces distribution. Under normal conditions of management m_{a1} and m_{b1} should be approximately equal.

m_{a2} and m_{b2} will give a comparison of the mean number of larvae migrating from clumps of faeces. (It is interesting to note that m_{a2} and m_{b2} should be directly related to the relative egg productions of the parasites in the hosts if the climatic conditions affecting hatching and survival are the same for both fields. It seems probable that the relative egg production should be expressed as a logarithm of egg counts (see Hunter & Quenouille, 1952). These comparisons would give a reasonable estimate of the available infective larvae, if the theoretical distribution fitted the

data fairly closely. Such information would be of considerable value but would need careful interpretation.

The implications of such an approach would have a profound effect on the method of sampling a pasture. To obtain an accurate estimate of the parameters it would be necessary to take a large number of samples and treat each sample separately, recording the number of larvae obtained from each. The importance of samples in which no larvae were obtained would be considerable, and any deficiency in recovery technique might have an overriding effect on the general result. With an efficient method of recovery of larvae, and a sample size expressed as an area of pasture related to the mean lateral movement of larvae, a good estimate might be obtained for limited areas of pasture. Intrinsic in the Neyman type of distribution is the postulate that the clumps of larvae must be distributed at random. This limits the technique to small areas and, as will be seen in a later section, limits the usefulness of such estimates of pasture infestation.

(*b*) *The relation of grazing pattern to distribution*

The grazing pattern of the host animal will affect the distribution of larvae upon the pastures, and must be considered here. Considering the whole pasture, a distribution of larvae approximating to Neyman's Contagious distribution will only be observed if the clumps of faeces are distributed at random, that is, if the animals defaecate at random. For the present purpose it is assumed that the times of grazing and defaecation are coincident. While it is realized that this may be an approximation only, the length of grazing time per day is sufficiently high for this assumption to be likely.

(i) *The distribution of faeces*

A survey of a field was made at 3-day intervals. Using a quadrant technique similar to the one used for botanical surveys, estimations of the number of freshly passed clumps of faeces was made. The number of these clumps occurring in various parts of the field on different days was recorded and the results presented in Fig. 2. It will be seen that areas where highest concentrations of freshly passed faeces occurred varied on different days. Observations of the flock suggested that these areas corresponded to the areas of highest grazing effort at that time.

(ii) *The distribution of sheep on a pasture*

Pasture infestation can never be considered static, and apart from continual migration and death of larvae there is, on a grazed pasture, continual recruitment of larval numbers as a result of contamination by faeces from infected hosts. Again, however, the infection of hosts may be assumed to be related to the pasture infestation, although, whether this relation is direct, continuous or discontinuous need not be considered here, and it is sufficient to state that within limits increase of host infection is closely linked with pasture infestation and vice versa. Thus the movements of sheep on a pasture will not only affect the distribution of larvae on the herbage but will also affect the number of larvae reaching a host in which they can develop and reproduce. Strictly these two approaches to

the problem cannot be divorced, and in considering pasture infestation it would not only be academic but also wrong in principle to consider only the distribution and disappearance of larvae in relation to the herbage, and ignore the factors which determine the survival and pattern development in the parasitic stage.

The obvious link between the two approaches is the behaviour of the grazing animal. Observations have been made on grazing flocks and this work is being continued to discover further details of flock behaviour in relation to parasitic infestation.

The general results obtained so far have great significance in considering parasitic infestations and the main points can be summarized here.

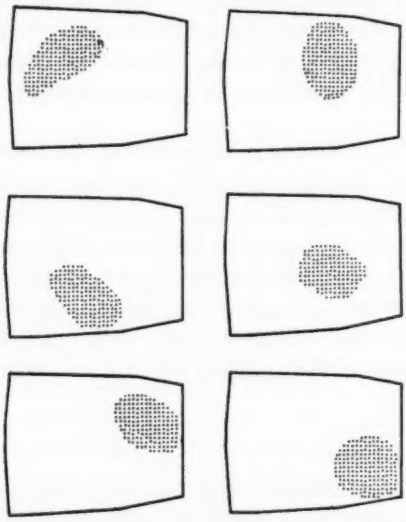

Fig. 2. The distribution of faeces over a field. The diagram is based on observations made at 3-day intervals, and the stippled portions show the areas of highest concentration of faeces.

Observation of sheep in fields has shown that on lowland farms, the sheep are not randomly spread, but move in a more or less well-integrated group. In this case the flock may be considered the unit of grazing. On hill farms the two main types of management (Crofton, 1949) give different patterns of behaviour. Where there is a controlled raking of the flock, the general picture is similar in some respects to that found on lowland farms. Here the flock is the unit of grazing, but this is made up of a number of subgroups which, while actually raking the hill, become more or less closely linked. In the type of hill management where the sheep are not raked up and down the hill daily the unit of grazing is not the flock but a small group which approximates to a ewe and its lambs.

The opportunity for studying lowland sheep is greater because the pastures are normally compact and well-defined areas. There are, however, some minor problems of observation, mainly concerned with the possibility of disturbing a flock while plotting the distribution of its members. Considerable help has been obtained by a series of aerial photographs supplied by the Air Ministry. These were taken

at heights of approximately 5000 ft. and have been obtained without flock disturbance (see Fig. 3).

From a series of photographs giving information about twenty flocks, it has been discovered that under what are presumably normal stocking conditions:

(1) The area occupied by a flock is rarely less than one-sixth and never more than one-third of the total pasture area. This gives a measure of compactness of the grazing unit.

(2) The area occupied per sheep is not proportional to the size of the pasture.

(3) The size of the flock is not directly proportional to the area occupied by the flock.

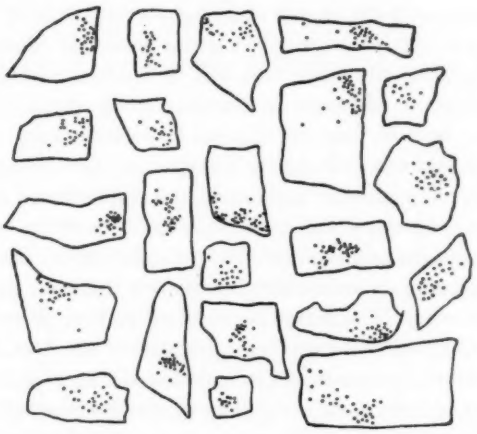

Fig. 3. The distribution of sheep in fields. The diagrams are taken from tracings of aerial photographs.

Obviously, from (2) and (3) the relation between individual members of a flock is not a simple one, and considerable and detailed work will be required to elucidate the factors influencing the compactness of a flock. However, these observations show that the flock must be considered the grazing unit. Thus the pasture can never be evenly grazed except over a relatively long period of time. As will be shown below, the clumps of faeces in which the larvae develop are not distributed randomly—a fact which might be deduced from the grazing pattern—so that the relationship between grazing and pick up must be a complicated one.

Preliminary observations suggest that the position of the flock in a field is related to the distribution of faeces. The flock is rarely if ever found grazing the area which has shown the highest concentration of faeces the previous day, and it is suggested that movement of the flock is more closely related to avoidance of contaminated areas than to lack of herbage.

Whatever the reason for the movement, the flock, if left long enough, will return to the area previously grazed, although the first area may not be completely regrazed. That is, the return may only involve part of the original area. If the return occurs in less than 6–7 days then there will be no chance of infection, the eggs deposited not having had time to reach the infective stage. If the return

coincides with the time of development of the infective stage then the rate of infection will be high and directly related to the proportion of the old area grazed. Maximum infection will occur if the new grazing area is coincident with the old one. If the time for return is longer than the time of development to the infective stage then the rate of infection will be related to the time of survival of infective stages. The longer the time before return, the lower the amount of infection.

The frequency of return will be related to the size of pasture, but the relation must be a complex one. Where the field is large in relation to flock size, the chances of return to an area will be small, but the chances will be greatly increased if pasture is poor and area grazed per sheep is large. Thus the chances of return must be considered in relation to the rate of stocking, not in terms of sheep per acre but in terms of sheep per unit of grazing. It seems probable, therefore, that the intervals between regrazing the same area will be approximately the same for any field (within limits) if the efficiency of stocking is the same. From the parasitic point of view there will be various degrees in overstocking. An extreme case would be where the field was sufficiently small to exactly contain a flock scattered in a normal manner, any smaller field causing a disturbance in flock behaviour. Where the flock occupies the whole area of the field there can only be interchange of position of individuals, and change within a small area. This will result in the sheep continually grazing an area which is infective from the time of development of the first infective stage. This latter is the usual picture given for any flock size, but it can be seen to be only a special case because of the flock behaviour and the movement of the sheep. It is possible to visualize the same result in a larger field, where the movement of the flock is rapid or where the grazing areas are the same for each circuit of grazing.

It will be seen that the observations throw some light on the increase of parasitic infections in a flock. More work is required but it is obvious that problems of stocking are complicated and that it is difficult to judge which is an ideal stocking policy. When this cannot be decided on the basis of number of sheep per acre, the main problems to be studied are concentrations of pasture infestation in relation to flock movement, and high rates of infection can occur in what might on other grounds be described as a lightly stocked field.

Apart from the broader aspects, these observations have considerable interest in relation to the estimation of larval infestation. If the larval populations are concentrated as a result of patterns of grazing, random samples will at best only give an estimate of the potential infection of a sheep grazing at random. The general result of pasture sampling will be to give estimates which are considerably lower than they should be if they are to be related to maximal uptake of larvae by a flock of sheep. Only a detailed knowledge of pasture infestation including not only numbers of larvae, but also areas of concentration, *and* a knowledge of the flock movements in the field, can be expected to give any reliable information about parasitic populations of pasture and host. Random samples from a pasture throughout the season will give estimates which are a reflexion of the flock infection. Maximum estimates from such samples will occur at times of peak infection of the hosts. Thus pasture samples taken at random can only give some

indication of the general level of infection of the grazing animals, and estimates of this can be obtained more efficiently by worm egg counts. In a previous paper, Crofton (1952) described the change of pasture infestation occurring on three farms. The differences in times of maximum infestation were noted, and it was shown that these times were relatively constant for any one farm over a period of 3 years, but the times differed between farms. It was tentatively suggested that stocking and management and time of lambing might account for this. The present work suggests that the figures obtained were merely reflexions of the parasite populations of the flocks. The constancy for years and the difference between farms has been shown to have some parallel in the parasite populations in lambs, but this will be discussed in more detail in a later paper.

III. THE USE AND LIMITATIONS OF PASTURE SAMPLING METHODS

In general the overall sampling of a pasture gives an inaccurate picture of the level of infestation because the larvae are not randomly distributed. Any such assessment can give little or no information about the potentialities of the pasture in relation to the infection of grazing sheep because the sheep do not graze randomly. Only in extreme cases is sampling of a whole field of any value; where high larval counts (more than 800 larvae/lb.) occur it can be assumed that there is high potentiality for infecting the definitive host but the possible rate of infection is very much higher than a mean count indicates. On the other hand, counts of 200–400 larvae/lb. may indicate a very high concentration of infective larvae in some areas of the pasture. The interpretation of the results obtained from such sampling from a whole pasture is extremely difficult, but a series of such results over a period of time gives some indication of the general trend of infestation and even the period of maximum infectivity may be determined. Such a series of samples taken throughout the year gives little or no greater information than may be deduced from a series of worm-egg counts carried out on faeces from the animals grazing the pasture, and the worm-egg count method is less laborious.

Theoretically the pasture sampling method could be used to determine if a pasture is free from infection, but lack of recovery of larvae is as difficult to interpret as a zero count in any sampling method. Increasing the number of observations will give more confidence in a zero count but in the case of pasture sampling, where the area of the samples is a very small part of the total area, the repetition of observations necessary to establish confidence in a zero result involves an impossible amount of labour. For most practical purposes it is sufficient to establish that the infestation of the pasture is very low.

In contrast with the general overall sampling of a field, sampling of selected areas may be of considerable use in determining the pattern of infestation if the selected areas are chosen in relation to the grazing habits of the particular flock under consideration. Moreover, by careful choice and limitation of the areas sampled, many of the difficulties of technique can be avoided. Selection of sampling area will also facilitate the application of the type of analysis suggested above (see §II, above), because areas can be chosen such that α and β can be given approximate values by direct observation.

Thus, techniques of estimating pasture infestation appear to be of relatively little value in making general surveys of parasite populations, systematic study of worm-egg output of the grazing hosts supplying as much information, more easily. In small scale but detailed studies of areas of pastures, the pasture sampling technique could be extremely useful. As has been suggested from preliminary observations on flock grazing habits a study of the infestation of small areas of pasture in different parts of a field is more likely to contribute to our knowledge of populations of parasites infecting flock animals than methods of estimating changes in gross populations, on the pasture or in the flock.

SUMMARY

1. Methods of sampling pastures for infective larvae are discussed.

2. An account is given of the methods of operation and identification of larvae from herbage.

3. The distribution of larvae on pastures is discussed in relation to choice of method of sampling and interpretation of results.

4. The significance of grazing patterns to distribution of larvae is considered.

5. An attempt is made to evaluate the use and limitations of pasture sampling methods.

The author is indebted to the Agricultural Research Council for financial assistance in the form of a special research grant.

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(*MS. received for publication* 28. IX. 1953.—Ed.)

TRICOTYLEDONIA GENYPTERI N.G., N.SP., A THREE-SUCKERED TREMATODE FROM THE RED LING, *GENYPTERUS BLACODES* BLOCH & SCHN.

By M. L. FYFE, M.Sc.

Department of Zoology, University of Otago, New Zealand

(With 5 Figures in the Text)

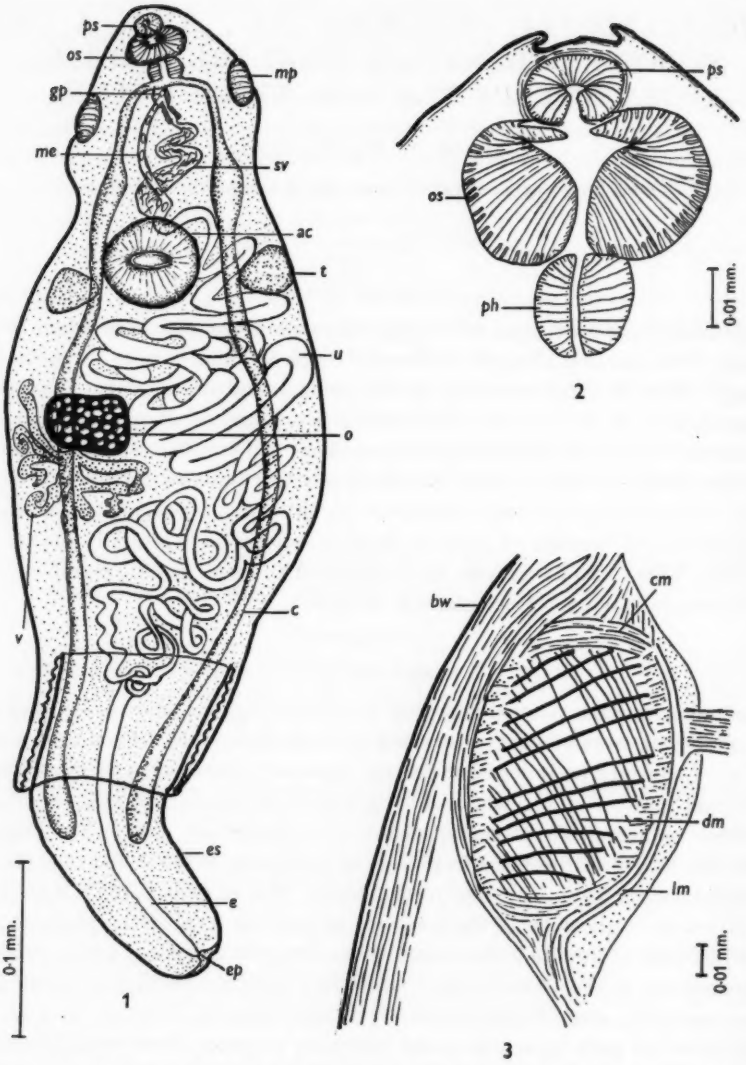
INTRODUCTION

A large number of specimens of an appendiculate Hemiurid were taken from the stomach of the red ling, *Genypterus blacodes* Bloch & Schn., caught in coastal waters of Otago, New Zealand, as many as 120 being found in one fish. In a personal communication H. W. Manter states that he has found adult specimens in the red ling and the conger eel and immature specimens in four other species of fishes, all from New Zealand waters. This Hemiurid has two unusual features: (1) a small definite sucker with a limiting membrane, anterior to the oral sucker, and (2) two conspicuous oval bundles of muscles forming muscle pads laterally placed in the fore-body. These two characters are considered to be of generic significance, and the trematode is named *Tricotyledonia genypteri*.

DESCRIPTION

Colour red with dark patches marking position of eggs in uterus; body elongate, oval, widest at level of ovary, tapering to both ends; very robust and muscular (Fig. 1). Length of body without ecsoma (measurements taken of nine preserved specimens) 3·90–5·10 mm.; width of body 1·05–1·98 mm.; ecsoma extended only in specimens subjected to pressure, about 1/3·5 length of body. Forebody very extensible; length of forebody in preserved specimens 0·930–1·282 mm., in living specimens greater than length of rest of body. Muscle pads (Fig. 3) embedded in the body wall on the sides of the forebody behind the level of the pharynx; longitudinal bundles of muscle fibres extend from the pads anteriorly to the oral sucker and posteriorly to the acetabulum, a transverse band connecting the anterior ends of the two pads; size of pads 0·260–0·316 mm. long by 0·167–0·186 mm. wide; muscle fibres of pads arranged in the following sequence from outside: one layer of longitudinal fibres, one layer of circular fibres, two layers of diagonal fibres at right angles to each other; outer longitudinal muscle layer continuous anteriorly and posteriorly with longitudinal muscles of body wall. The muscle pads and the muscle fibres attached to them contribute greatly to the extensibility of forebody.

Transverse diameter of oral sucker 0·298–0·419 mm.; diameter of acetabulum 0·595–0·837 mm.; sucker ratio in all specimens 1:2. Preoral sucker immediately in front of oral sucker (Fig. 2), bilobed with rounded fleshy lappet projecting in front; transverse diameter of preoral sucker 0·112–0·205 mm. Preacetabular pit half way between acetabulum and genital pore; pit leads into narrow duct opening



(Figs. 1–3 were drawn with the aid of a camera lucida. Abbreviations: *ac*, acetabulum; *bw*, body wall; *c*, caecum; *cm*, circular muscles; *dm*, diagonal muscles; *e*, excretory vesicle; *es*, ecsoma; *ep*, excretory pore; *ev*, ejaculatory vesicle; *gp*, genital pore; *h*, hermaphrodite duct; *lm*, longitudinal muscles; *me*, metraterm; *mp*, muscle pad; *o*, ovary; *od*, oviduct; *os*, oral sucker; *pg*, prostate gland; *ph*, pharynx; *pp*, pars prostatica; *ps*, preoral sucker; *sh*, shell gland; *sr*, seminal receptacle; *ss*, sinus sac; *sv*, seminal vesicle; *t*, testis; *u*, uterus; *v*, vitellaria.)

Fig. 1. *T. genypteri*. Ventral view. Scale = 0·1 mm. Fig. 2. Horizontal section of anterior end showing preoral sucker, oral sucker and pharynx. Scale = 0·01 mm. Fig. 3. Horizontal section of muscle pad. Scale = 0·01 mm.

into glandular cavity. Pharynx 0·167–0·186 mm. long by 0·139–0·186 mm. wide. Caeca enter ecsoma in living specimens when ecsoma is extended.

Genital pore slightly behind and to the right of pharynx. Male and female genital ducts join to form short hermaphrodite ducts (Fig. 4). Two testes at level of posterior part of acetabulum, 0·260–0·335 by 0·316–0·372 mm.; seminal vesicle extending forward from acetabulum. Pars prostatica short with large prostate

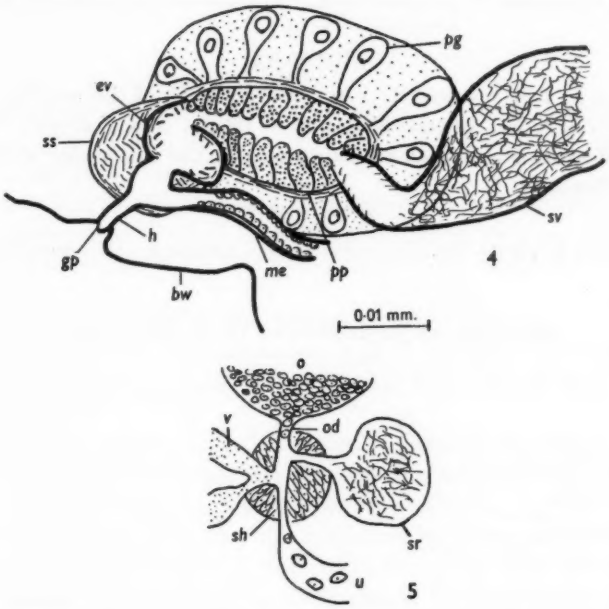


Fig. 4. Horizontal section through genital pore showing male and female ducts. Scale = 0·01 mm. Drawn with the aid of a camera lucida.

Fig. 5. Diagram of ovarian complex. Abbreviations as in Figs. 1–3.

glands. Sinus sac small, spherical, enclosing end of pars prostatica opening into ejaculatory vesicle, terminal part of metraterm and short hermaphrodite duct (Fig. 4). Genital papilla sometimes present. Ovary well behind testes, on right side; size 0·298–0·316 by 0·461–0·539 mm., long axis transverse; seminal receptacle (Fig. 5) large. Vitellarium with seven lobes ranging from short and thick to long and thin; uterus with numerous coils posterior to ovary, coils do not enter ecsoma. Laurer's canal lacking. Eggs 12–13 by 8–9μ.

Generic diagnosis of T. genypteri. Fairly large appendiculate Hemiuridae with small sucker anterior to oral sucker and two oval muscle pads laterally placed in the forebody. Preacetabular pit present. Vitellaria small with seven finger-like lobes. Pars prostatica short, enters ejaculatory vesicle in sinus sac; sinus sac small, muscular. Laurer's canal lacking.

DISCUSSION

T. genypteri does not fit into any of the subfamilies of Hemiuridae as set out by Dawes (1946, p. 90), since the presence or absence of a preoral lip (not a sucker) is used as a means of separating the subfamilies. If the present key is to stand, a new subfamily should be created for those forms with a sucker in front of the mouth, but this can be avoided if the classification is based on the character of the vitellaria only, in which case *T. genypteri* would be placed in the subfamily Sterrhurinae.

SUMMARY

T. genypteri n.g., n.sp., is described from the stomach of the red ling, *Genypterus blacodes* Bloch & Schn., in coastal waters of Otago, New Zealand. This genus is characterized by having a small sucker anterior to the oral sucker and two muscle pads in the forebody.

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NEW SPECIES OF LARVAL MITES FROM INDIA (ACARINA: TROMBICULIDAE)

By T. B. SINHA, M.Sc., F.Z.S.*

Zoology Department, Allahabad University

(With 4 Figures in the Text)

INTRODUCTION

The specimens described in this paper were collected in Assam by Dr M. L. Roonwal during World War II, and were sent to me for identification by Dr S. L. Hora, Director, Zoological Survey of India.

I am grateful to Prof. H. R. Mehra, Professor of Zoology, Allahabad University, for his able guidance, to Dr Hora for sending me the material and to Dr Charles D. Radford for his opinion on the species described in this paper.

Mehracula roonwali n.g., n.sp.

This genus has been named in the honour of Prof. H. R. Mehra under whose guidance the work was carried out, and the species has been named after its collector, Dr M. L. Roonwal.

The body of this species, like that of other species of the genus *Mehracula*, is marked with wavy and somewhat straight annulations. The dorsal scutum (Fig. 1*a*) is punctate and rectangular; it is much wider that it is long and its anterior edge is concave and its posterior edge convex, the lateral edge being concave. The antero-lateral spines are closely barbed. The antero-median spine is also closely barbed and its base is situated 7·2μ behind the anterior edge of the scutum. The postero-lateral spines are thickly barbed and their bases are located in the postero-lateral angles of the scutum and just anterior to the line of the sens. The sensilla is flagelliform with only 4 small barbs in its apical part. There are 2 barbed humeral spines. There are 44 dorsal spines arranged in rows of 10, 12, 10, 8 and 4. Ocular plates are present, adjacent to the postero-lateral spines.

On the venter (Fig. 1*b*) of the larva there is a pair of setae between coxae 1 and these are longer than the other pair situated between the coxae 3. Behind coxae 3 there are 16 short, simple spines arranged in rows of 8 and 8. Behind these again there are 22 spines which are like those found on the dorsum of the larva and are arranged in rows of 2, 6, 4, 4, 4 and 2. Each of the coxae bears a single spine.

All the legs have seven segments, and each of the tarsi has a weak empodium and a pair of claws. The third pair of legs, each of which is 284·3μ long, is the longest, the legs of the second pair being 212·2μ and those of the first pair 234μ long.

The palpal claw is tridentate and the first, second and third palpal setae are nude, while the fourth is laterally barbed and the fifth is nude.

The galeal seta is barbed. Each chelicera (Fig. 1*c*) has a minute, knoblike tooth in the subapical region.

* Jr. N.I.S. Research Fellow.

*Standard data.** AW 57·6μ, PW 68·4μ, SB 28·8μ, ASB 21·6μ, PSB 7·2μ, A–P 23μ, AM 39·6μ, AL 39·6μ, PL 43·2μ, Sens. 61·2μ, DS 39·6μ, Humerals 43·2μ, SD 28·8μ.

Host. Tree shrew, *Tupaia belangeri* (Wag).

Locality. MS 34 on Manipur–Taru Road, Assam, 14 November 1945, Dr M. L. Roonwal, Coll. No. R 2/14.11.45.

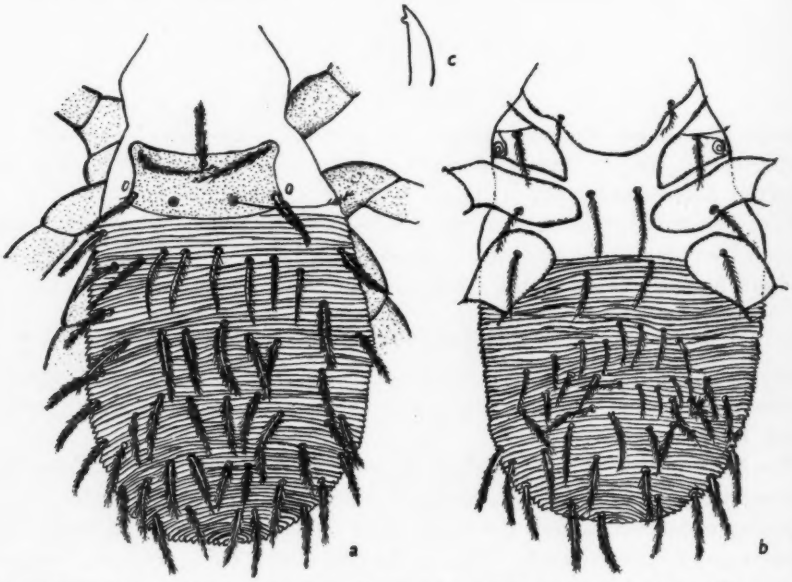


Fig. 1. (*a*) Dorsal view of *Mehracula roonwali* n.g., n.sp. × 240. (*b*) Ventral view of *M. roonwali* n.g., n.sp. × 240. (*c*) Chelicera of *M. roonwali* n.g., n.sp. × 360.

This species is close to *Mehracula radfordi* n.sp., but the shape of its dorsal scutum is different, as are also its measurements and the arrangement of its spines. This species is also close to *Trombicula scutellaris* Nagayo, but it has fewer barbs on the apical half of the sensillae and no minute barbs on the basal half of the sensillae. The anterior edge of the dorsal scutum is more concave in this species than it is in *T. scutellaris*.

Genotype. Mehracula roonwali n.g., n.sp.

Genotype. Slide No. R 2/14.11.45, Zoological Survey of India (MS 34 on Imphal–Taru Road, Assam, 14 November 1945, Dr M. L. Roonwal, specimen in a ring).

Paratype. On Slide No. R 2/14.11.45, many specimens, with above data.

Mehracula radfordi n.sp.

This species is named after Dr Charles D. Radford who has contributed so much to our knowledge of the Acarina.

The body of the larva shows very prominent, somewhat wavy annulations. The dorsal scutum (Fig. 2*a*) is more or less rectangular in shape and is punctate, with

* For key to abbreviations see, p. 337.

its anterior edge practically straight; the lateral edge has a slight concavity in the middle and the posterior edge is more or less straight. The antero-lateral spines are barbed. The antero-median spine is somewhat bushy and its base is placed some distance behind the anterior edge of the scutum. The postero-lateral spines are branched and their bases are situated in the postero-lateral angles of the scutum and are also posterior to the bases of the sensory spines. The pseudostigmatic

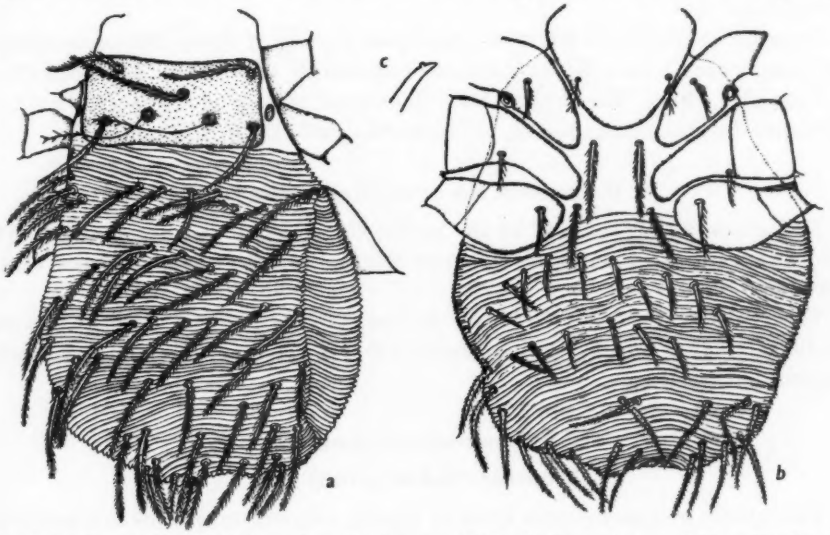


Fig. 2. (*a*) Dorsal view of *Mehracula radfordi* n.sp. × 240. (*b*) Ventral view of *M. radfordi* n.sp. × 240. (*c*) Chelicera of *M. radfordi* n.sp. × 360.

organs are flagelliform with a few barbs in the apical half. There are two barbed humeral spines. On the dorsum there are forty-six spines arranged in rows of 8, 2, 10, 10, 4, 6, 4 and 2. Ocular plates are present on each side of the dorsal scutum and a little above the level of the postero-lateral spines. All the legs have seven segments and each tarsus has a pair of claws and a simple empodium. There is no mastitarsala on tarsus 3.

The venter of the larva (Fig. 2*b*) shows a pair of long spines between the coxae 1 and another pair of shorter spines between coxae 3. Caudad to this there are two rows of short, simple spines, arranged in rows of 10 and 6, and further behind there are eighteen stout spines like the one present on the dorsum and these are arranged in rows of 8, 6 and 4. Each of the coxae has a single spine.

The palpal claw is tridentate. All the palpal spines are nude and the galeal seta is also nude. The chelicera (Fig. 2*c*) has a single minute, rounded, knob-like tooth in the subapical region.

Standard data. AW 64·8 μ, PW 70·2 μ, SB 28·8 μ, ASB 28·8 μ, PSB 18 μ, A–P 28·2 μ, AM 54 μ, AL 39·6 μ, PL 43·6 μ, Sens. 56·6 μ, DS 43·2 μ, Humerals 41·4 μ, SD 46·8 μ.

Hosts. The crab-eating mongoose, *Herpestes urva* (Hodgs.) and the squirrel, *Dremomys macmillani*.

Localities. Madbung village, *off* Kanglatongli, camp MS 117, Assam, 1 December 1945, Dr M. L. Roonwal, Coll. No. R 2/1.12.45; MS 117 on Dimapur–Imphal Road, Assam, 12 November 1945, Dr M. L. Roonwal, Coll. No. R 9/12.11.45.

This species is close to *Trombicula scutellaris* Nagayo, 1920, but the shape of its dorsal scutum is different and the structures on the scutum are shorter in this species. Its annulate body differentiates it from all the known species of the genus *Trombicula*.

Holotype. Slide No. R 2/1.12.45, Zoological Survey of India (Madbung village, *off* Kanglatongli, camp MS 117, Assam, 1 December 1945, Dr M. L. Roonwal).

Paratypes. Slide No. R 9/12.11.45, Zoological Survey of India (MS 117, on Dimapur–Imphal Road, Assam, 12 November 1945, Dr M. L. Roonwal).

Diagnosis of the genus **Mehracula** n.g.

This genus is characterized by the presence of annulate markings on the body and a very minute, blunt, rounded, knob-like tooth in the subapical region of the chelicera.

The genus is close to *Trombicula*, Berlese, but its shape is different and the cheliceral tooth is rounded in this genus, while it is sharp, saw-like and backwardly directed in *Trombicula*.

Genus *Trombiculindus* Radford, 1948

Trombiculindus pruthi n.sp.

This species was named after Dr H. S. Pruthi, who encouraged me to take up the study of this fascinating group.

The dorsal scutum (Fig. 3*a*) is more or less trapezoidal in shape and its anterior edge is slightly concave; its lateral edge is oblique and its posterior edge is slightly convex. The antero-lateral spines are barbed. The antero-median spine is barbed and is situated some distance behind the anterior edge of the scutum. The postero-lateral spines are leaf-like and are about $3\frac{3}{4}$ times longer than they are wide. The bases of these spines are situated just anterior to the postero-lateral angles of the scutum and anterior to the bases of the pseudostigmatic organs. The sensory spine (one missing) is nude. There are two leaf-like humeral spines. On the dorsum there are 40 leaf-like spines arranged in rows of 10, 8, 8, 6, 6 and 2. Ocular plate is not clearly seen. All the legs have seven segments and each tarsus has a pair of claws and a simple empodium. There is no mastitarsala on tarsus 3.

The venter of the larva (Fig. 3*b*) shows a pair of long spines between coxae 1 and another slightly smaller pair between coxae 3. Posterior to these are three rows of 16 simple spines arranged in rows of 8, 4 and 4, and then follow 12 leaf-like spines, arranged in rows of 2, 6 and 4 which resemble those present on the dorsum. Each of the coxae has a single spine.

The palpal claw is tridentate. All the palpal spines are nude. The second palpal spine is very long and measures 21·6 μ in length. The galeal seta is barbed.

Standard data. AW 72 μ, PW 82·8 μ, SB 36 μ, ASB 25·2 μ, PSB 7·2 μ, A–P 14·4 μ, AM 50·4 μ, AL 54 μ, PL 50·4 μ, Sens. 36 μ, DS 28·8 μ, Humerals 43·2 μ, SD 32·4 μ.

Hosts. The crab-eating mongoose, *Herpestes urva* (Hodgs.) and the squirrel, *Dremomys macmillani*.

Localities. Madbung Village, *off* Kanglatongli, camp MS 117 Assam, 1 December 1945, Dr M. L. Roonwal, Coll. No. R 2/1.12.45, MS 117, on Dimapur–Imphal Road, Assam, 12 November 1945, Dr M. L. Roonwal, Coll. No. R 9/12.11.45.

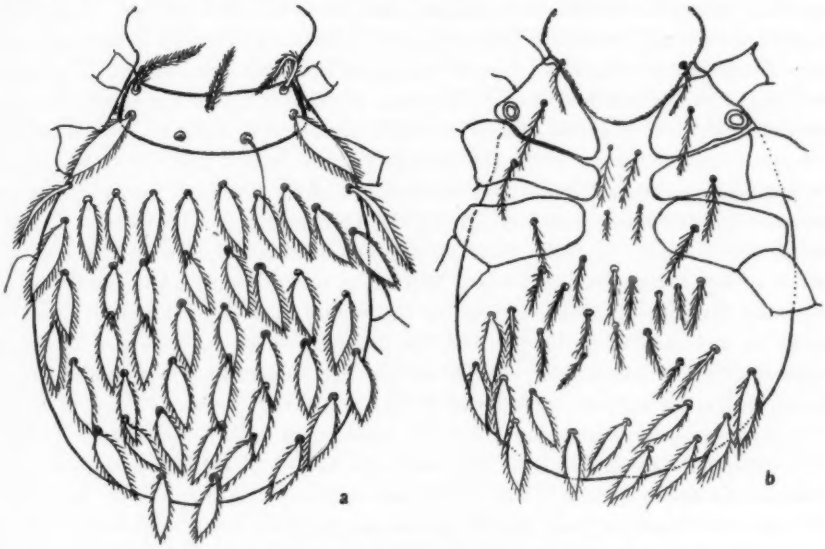


Fig. 3. (*a*) Dorsal view of *Trombiculindus pruthi* n.sp. × 240.
(*b*) Ventral view of *T. pruthi* n.sp. × 240.

This species is close to *Trombiculindus squamifera* Womersley (1952), but it has 12 foliate spines on the venter of the abdomen while *T. squamifera* has 4 to 6. Further, the postero-lateral spines of *T. pruthi* are about $3\frac{3}{4}$ times longer than they are wide, while those of *T. squamifera* are $3\frac{1}{2}$ times longer than they are wide.

Holotype. Slide No. R 2/1.12.45, Zoological Survey of India (Madbung village *off* Kanglatongli, camp MS 117, Assam, 1 December 1945, Dr M. L. Roonwal).

Paratype. Slide No. R 9/12.11.45, Zoological Survey of India, MS 117, on Dimapur–Imphal Road, Assam, 12 November 1945, Dr M. L. Roonwal.

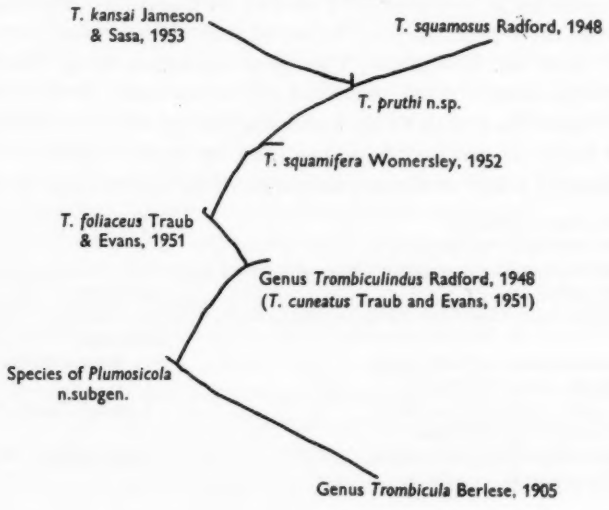
The following is a key to the world species of *Trombiculindus* Radford (1948):

1.	All ventral setae feathered.	2
	All ventral setae not feathered.	3
2.	Postero-lateral spines about three times as long as wide, with small barbs; sensillae with basal barbs.	**cuneatus** Traub & Evans, 1951
	Postero-lateral spines less than twice as long as wide, with reticulations; sensillae nude basally.	**foliaceus** Traub & Evans, 1951
3.	Postero-lateral spine without barbs.	**squamosus** Radford, 1948
	Postero-lateral spine with barbs.	4
4.	With two pairs of humeral setae.	**kansai** Jameson & Sasa, 1953
	With one pair of humeral setae.	5
5.	Four to six foliate spines on venter.	**squamifera** Womersley, 1952
	Twelve foliate spines on venter.	**pruthi** n.sp.

Speciation in Trombiculindus *Radford*, 1948

The three species of the genus *Trombicula*: *T. plumosa* Radford, 1953, *T. intermedia* Nagayo, 1920 and *T. hastata* Gater, 1932, are closely related to one another and stand apart from all the other species, so that they form a natural group. In species of this group the dorsum and the postero-lateral have plumose spines, an important character which distinguishes them from all other species. A new subgenus of the genus *Trombicula* Berlese should therefore be created to accommodate them. For this new subgenus I propose the name *Plumosicola* n.subgen., *T. plumosa* Radford, 1953, being the type of it. It seems probable that during speciation some member of the above group of species might have given origin to *Trombiculindus cuneatus* Traub & Evans, 1951, the normal postero-lateral and the spines on the dorsum of *Trombicula* Berlese first becoming slightly flattened and plumose and then strongly flattened (leaf-like), giving rise to *Trombiculindus cuneatus* Traub & Evans, 1951. I also consider that the number of foliate spines on the venter of species of the genus *Trombiculindus* had great evolutionary value and that the lesser the number of foliate spines on the venter, the more lowly evolved the species is. If this is taken for granted, the question of the speciation in *Trombiculindus* Radford becomes clear, and the species can be arranged in ascending evolutionary order. Thus *T. cuneatus* Traub & Evans, 1951, *T. foliaceus* Traub & Evans, 1951, *T. squamifera* Womersley, 1952, *T. pruthi* n.sp. and *T. squamosus* Radford, 1948, represent the earlier condition, and the height of evolution is reached by *T. kansai* Jameson & Sasa, 1953, which has eighteen foliate spines on the venter.

T. kansai Jameson & Sasa and *T. squamosus* Radford need further explanation, because the former has two pairs of humeral spines, while in the latter all the foliate spines and postero-lateral spines are devoid of barbs, but have punctae. It seems that during evolution, two branches evolved near the origin of *T. pruthi* n.sp., one branch giving rise to *T. squamosus* Radford and the other to *T. kansai* Jameson & Sasa. The evolution of the species can be represented as follows:


T. kansai Jameson & Sasa, 1953
T. squamosus Radford, 1948
T. pruthi n.sp.
T. squamifera Womersley, 1952
T. foliaceus Traub & Evans, 1951
Genus Trombiculindus Radford, 1948 (T. cuneatus Traub and Evans, 1951)
Species of Plumosicola n.subgen.
Genus Trombicula Berlese, 1905

A note on the genera Trombiculindus *Radford and* Fonsecia *Radford*

The genus *Trombiculindus* was described by Radford in 1948 with *T. squamosus* as genotype. Later on Traub & Evans (1951) added to this genus two more species, *T. cuneatus* and *T. foliaceus*. Wharton, Jenkins, Brennan, Fuller, Kohls & Philip (1951) treated this genus as a subgenus of *Trombicula* Berlese (1905), and Womersley (1952) described another species, *T.* (*T.*) *squamifera*, and followed Wharton *et al.* in treating this genus as a subgenus, although his reasons for doing this were different from those of Wharton *et al.* Recently, Jameson & Sasa (1953) have added yet another species, *T.* (*T.*) *kansai* and have followed Wharton *et al.* and Womersley in treating this genus as a subgenus.

Womersley (1952), although he described this genus as a subgenus, said that the leaf-like spines are seasonal and are developed by the animal to escape desiccation. Neither I, nor Radford (1953*a*) can, however, understand why a particular species should, in order to escape desiccation, develop such characters, while other allied species do not, although they live under similar conditions. I therefore consider that *Trombiculindus* Radford should be treated as a genus and not as a subgenus. The main reason for this taxonomic decision is that the leaf-like postero-lateral spines are invariably present in all the known species of this genus and also in the species *T. pruthi* n.sp. described in this paper.

The genus *Fonsecia* Radford (1942) has also been treated as a subgenus by Wharton *et al.* (1951). I am, however, opposed to such a view, because there is not a single example known which shows an intermediate stage between the normal setation and the peg-like setation of the postero-lateral spine which is characteristic of this genus. I, therefore, feel convinced that this genus should be treated not as subgenus but as a genus. Radford (1953*a*) agrees with me that both *Trombiculindus* and *Fonsecia* should be genera.

Genus *Walchia* Ewing, 1931

Walchia manipurensis n.sp.

The dorsal scutum (Fig. 4*a*) is punctate and is pentagonal in shape; its anterior edge is slightly depressed in the middle; its lateral edge is concave, the concavity being anterior to the pseudostigmatic organs; its posterior edge is convex and bluntly pointed. The antero-lateral spines are barbed. The antero-median spine is absent. The postero-lateral spines are barbed and are situated posterior to the postero-lateral angles of the scutum. The leaf-like pseudostigmatic organs are beset with fine hairs and are attached to the scutum by a fine peduncle. Anterior to each of the pseudostigmatic organs there is a ridge. There are two barbed humeral spines. On the dorsum there are 34 simple spines, arranged in rows of 6, 10, 6, 6, 4 and 2. The ocular plate is absent. With the exception of the first leg, which has seven segments, the legs have only six segments each. All the tarsi have three claws, the median claw being feebler than the lateral ones.

The venter of the larva (Fig. 4*b*) shows a pair of long spines in front of coxae 1 and another slightly smaller pair of spines between coxae 3. Posterior to these

there are 46 smaller spines arranged in rows of 10, 2, 8, 10, 6, 6 and 4. Each of the coxae has a single spine.

All the palpal spines are nude.

Standard data. AW 21·6 μ, PW 28·8 μ, SB 21·6 μ, ASB 18 μ, PSB 43·2 μ, A–P 36 μ, PL 39·6 μ, Sens. 25·2 μ, DS 28·8 μ, Humerals 21·6 μ, SD 61·2 μ.

Host. Bandicoot, *Bandicoota bengalensis bangalensis* (Gr. & Hardev).

Locality. Kanglatongli, camp site, Assam, 9 October 1945, Dr M. L. Roonwal, Coll. No. R 6/9.10.45.

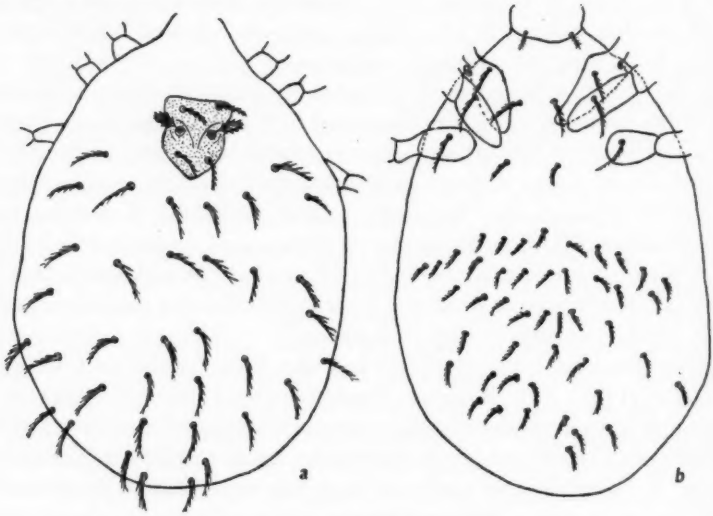


Fig. 4. (*a*) Dorsal view of *Walchia manipurensis* n.sp. × 180.
(*b*) Ventral view of *W. manipurensis* n.sp. × 180.

This species is close to *Walchia glabrum* (Walch), but the tarsal claws do not show the great disparity shown by those of *W. glabrum*. This species also differs from *W. glabrum* in the number of spines and in its measurements.

Holotype. Slide No. R 6/9.10.45, Zoological Survey of India, Kanglatongli, camp site, Assam, 9 October 1945, Dr M. L. Roonwal.

Paratype. Slide No. R 6/9.10.45, Zoological Survey of India, Kanglatongli, camp Site, Assam, 9 October 1945, Dr M. L. Roonwal.

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Key to the abbreviations used

AW Distance between the bases of the antero-lateral spines.
PW Distance between the bases of the postero-lateral spines.
SB Distance between the bases of the sensilla.
ASB Distance from the anterior margin of the scutum to the level of the sensillary bases.
PSB Distance from the posterior margin of the scutum to the level of the sensillary bases.
A–P Distance between the bases of the antero-lateral and postero-lateral scutal setae.
AM Length of the antero-median seta.
PL Length of the postero-lateral seta.
AL Length of the antero-lateral seta.
Sens. Length of the sensilla.
DS Length of the seta on the dorsum of the body.
Humerals Length of the humeral seta.
SD = ASB + PSB.

* Could not be consulted in original.

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THE DURATION OF RESIDUAL IMMUNITY FOLLOWING SPONTANEOUS CURE OF *PLASMODIUM BERGHEI* IN RATS

By C. C. DRAPER

Ross Institute of Tropical Hygiene, London School of Hygiene and Tropical Medicine

(With 1 Figure in the Text)

The inoculation of blood containing *Plasmodium berghei* into rats gives rise to an acute infection. In young rats this is very often fatal, but of mature rats the majority succeed in controlling the infection after several weeks, which then passes into a latent stage and is ultimately spontaneously eradicated. The importance of the spleen in the genesis and maintenance of acquired immunity was demonstrated by Galliard & Lapierre (1950) and has since been confirmed by other workers. Splenectomy before inoculation causes the subsequent infection to run a fulminant course, whereas if this is done during the latent stage a chronic infection results with frequent parasitic recrudescences.

During the latent stage of the infection, intact rats are highly immune to homologous or heterologous superinfection, even massive intracardiac or intraperitoneal inoculations of infected blood causing only a transient parasitaemia (Vargues & Fabiani, 1951; Fabiani & Vargues, 1951). This acquired immunity has been shown to persist after spontaneous eradication of the infection, and so is not due entirely to premunition. The time at which spontaneous cure occurs, and thus when one is able to test the strength of true residual immunity to reinfection, varies with the strain of rat and of *P. berghei* used.

Corradetti (1950, 1952) performed splenectomies on rats at varying intervals after recovery from the acute stage of infection and found that spontaneous eradication, as shown by the lack of a recrudescence, occurred 2–3 months after the day of inoculation. There was complete immunity to homologous reinfection until at least 7 months after inoculation, the latest date at which it was attempted, showing that residual immunity persisted for at least 4–5 months. This was reduced by splenectomy of recovered rats; reinfection then produced a chronic infection shown to persist for at least 8 months in one rat, although another rat succeeded in eradicating the infection after 1 month, demonstrating that there is also some extra-splenic mechanism maintaining immunity.

Black (1951), by means of splenectomies and of subinoculations from infected rats into clean mice, showed that spontaneous cure occurred between 2 and 3 months after inoculation. On the other hand, Ramakrishnan & Satya Prakash (1951), using subinoculations into mice, found that spontaneous cure did not occur until about 6 months after inoculation; a complete residual immunity to homologous reinfection was present for at least another 4 months.

The work of Zuckerman & Yoeli (1951) and Zuckerman (1953) has shown that

a comparable response occurs in voles: spontaneous cure occurs 1–2 months after inoculation, and a strong residual immunity is present for at least a further 3 months, which is much reduced by splenectomy.

The purpose of this work was to test the degree of residual immunity in rats at periods after recovery longer than those previously described.

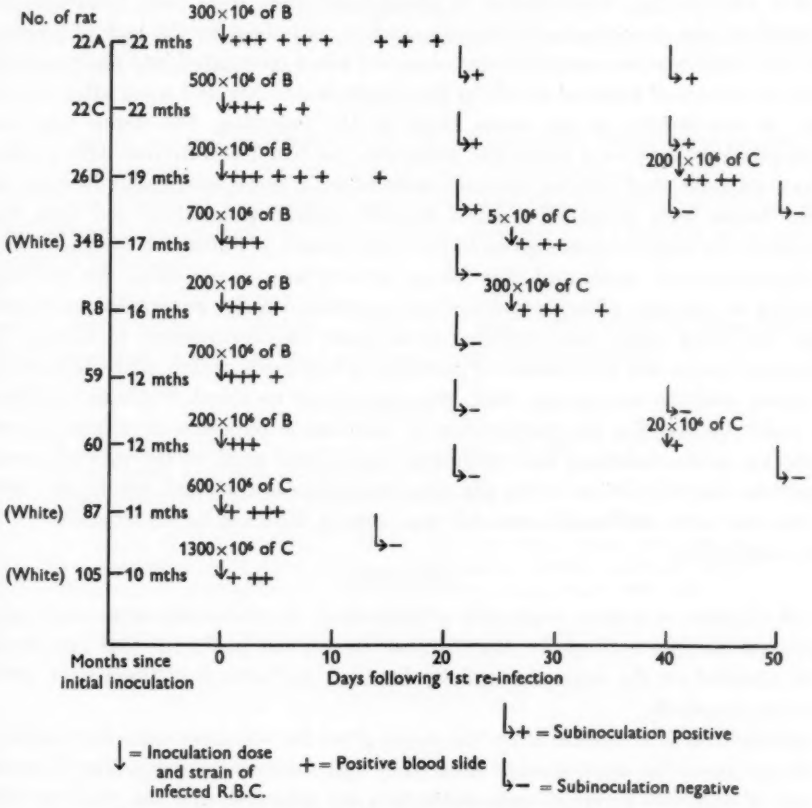

Fig. 1. Reinfections of rats recovered from *P. berghei*.

MATERIAL AND METHODS

The majority of rats used were black and white hooded rats. A few white rats were also studied, and these have been noted in Fig. 1. The rats were infected at the age of 3–4 months by intraperitoneal inoculation of blood infected with *P. berghei*. Each rat received either of two strains maintained in the Ross Institute. These, known as the Gevei and S.P. 28 strains, differed in their gametocyte production but otherwise were very similar: there was an initial mortality of about 10% in the inoculated rats; the survivors succeeded in controlling the acute stage of the infection in 2–3 weeks, and it then passed into the latent stage. Serial subinoculations from sixteen rats were done at different intervals by taking 0·1–0·2 ml. of tail blood and injecting it into susceptible white mice. By this means it was found

that although spontaneous eradication of the infection could occur in a few by $2\frac{1}{2}$ months after inoculation, the average period was about 4 months.

For the reinfections two heterologous strains of *P. berghei* were used which had recently (1953) been isolated in the Belgian Congo and maintained at the Ross Institute by passage through hamsters. These, known as the B (S.P.180) and C (S.P.148) strains, were similar in physiology; they were both somewhat less virulent to rats than strains previously studied, as judged by the lack of fatalities and the relatively low parasitaemias observed when inoculated into the rats which acted as donors of infected blood for the reinfections. About 1 week after inoculation, at the height of the acute stage of the infection, the donor rats were exsanguinated by means of cardiac puncture, the blood was diluted with a saline-citrate mixture, and varying amounts were injected intraperitoneally into the test rats. Doses were given of from 5 to 1000 million parasitized red cells, thus repeating the massive doses given in the experiments of Fabiani & Vargues (1951).

Giemsa-stained thick and thin blood smears were made from the test rats, starting on the day after reinfection and continued daily, except for week-ends, until the thick smear was negative on at least two consecutive occasions. The criterion for this was the absence of parasites in fifty fields, which, with the thickness of smear and the microscope used, was equivalent to about 0·05 mm.^{3} of blood. In order to test for the persistence of inoculated parasites in submicroscopic densities, subinoculations into mice were made from some of the rats at varying intervals after reinfection, using the same technique as described previously. Some of the rats were reinfected a second time using a different strain from that of the first reinfection.

RESULTS

In all thirteen rats were reinfected at periods of 10–22 months after their initial inoculations. The findings in nine of these are set out in Fig. 1, four rats having been omitted for the sake of simplicity because the findings in them were similar to those in others.

Subtraction of 4 months from the figure given for the time since inoculation of each rat gives the approximate time since spontaneous recovery. The doses and strain of parasites given at each reinfection are recorded, and the resulting parasitaemia is expressed as the number of days on which parasites were seen, using the criterion noted above.

It can be seen that the duration of parasitaemia is less affected by the size of the inoculum than by the period of recovery of the rat. At one extreme is rat 22A, which after reinfection showed a parasitaemia for nearly 3 weeks; this attained a maximum of 10% by the fourteenth day, and subinoculations showed the persistence of parasites until at least the fortieth day, when the experiment had to be terminated. Rat 22C responded similarly, although the overt parasitaemia was less in degree and in duration. At the other extreme are rats 60, 87 (and its unrecorded companions 85 and 89), and 105: in these the parasites inoculated at reinfection persisted only for a few days; the blood film showed evidence of active schizogony but the numbers of circulating parasites started to decrease from the second day after the reinfection.

Intermediate grades of response were shown by rats 26D (and its companion 26C), 34B (and its companion 34A), R8 and 59. A second reinfection of some rats with a different strain, even in comparatively small doses, produced a parasitaemia, showing that complete immunity had not been restored by the stimulus of the first reinfection.

As far as these results go they suggest that the residual immunity of rats after spontaneous cure is strong, but it is not complete and shows signs of waning at 15–18 months after cure.

SUMMARY

1. In intact rats infected with *P. berghei* spontaneous eradication of infection has occurred by about 4 months after the day of inoculation.

2. These recovered rats show a strong residual immunity to reinfection with a heterologous strain, but this has begun to wane by 15–18 months after cure of the initial infection.

I owe many thanks to Miss W. J. Wall and Miss J. Wrugh of the Ross Institute for maintenance of the rats and assistance with the blood films.

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THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE SIZE OF THE CYSTS OF *ENTAMOEBA INVADENS* RODHAIN, 1934

By ELSPETH W. McCONNACHIE

The Molteno Institute, University of Cambridge

(With 3 Figures in the Text)

INTRODUCTION

In order to obtain material for an *in vitro* study of *Entamoeba invadens*, several strains growing with simplified bacterial flora were produced by a method to be described elsewhere. These included strain A, growing in the presence of a saccharose-fermenting strain of *Bacterium coli* (designated 15) and an unidentified bacterium, organism *x*, and strain M, growing in the presence of a lysozyme-sensitive strain of *Bacillus megatherium* and organism *x*. Strain A was derived from cysts formed in a strain of *E. invadens* originally isolated from a reptile by Prof. J. Rodhain, and obtained by the Molteno Institute from Dr L. Lamy, and strain M was derived from cysts formed by strain A. The two strains, therefore, had a common origin, the only difference between them being that *Bacterium coli* 15 was present in the flora of strain A, and *Bacillus megatherium* in that of strain M, while organism *x* was common to both.

During routine examinations of cultures of strains A and M growing in diphasic and liquid nedia containing starch—inspissated horse-serum covered with Ringer-eggwhite (HSre + S of Dobell & Laidlaw, 1926) and liquid horse serum diluted with Ringer's solution (hs + S)—it was noticed that the cysts formed in the cultures of strain M appeared to be larger than those in the cultures of strain A. Confirmation of this observation was sought from measurements of the cysts formed in the two strains, and the data obtained were analysed in order to discover whether these strains of *E. invadens* comprised more than one race, as determined by cyst size.

EXPERIMENTAL METHODS AND RESULTS

The diameters of the cysts were measured by means of an ocular micrometer, one division of which was equivalent to 1·0μ. Cysts were stained with Lugol's iodine solution before being measured, and the preparations were sealed with wax to reduce evaporation. Dobell & Jepps (1918) found that there was no appreciable difference in the size of cysts of *E. histolytica* measured in saline or iodine solution, and the present measurements may therefore be considered to represent those of living cysts. These workers also observed that very few of the cysts of *E. histolytica* were perfectly spherical, and this was confirmed with the cysts of *E. invadens*, the majority of which were approximately spherical or ovoid. The diameter of each cyst was expressed as the mean value of its long and short axes, and the few cysts which were very irregular in shape were not measured. A few supernucleate cysts

(with five to eight nuclei) were observed in nearly all the cultures, and occurred in approximately the same numbers in the two strains; as these cysts tended to be larger than the normal 4-nucleate cysts, their measurements are not included in the present data. The diameters of 1000 cysts of strain A and 1000 cysts of strain M formed in HSre and hs medium were measured, the cysts being obtained in each instance from four separate cultures. The data obtained are shown in Table 1, and a statistical analysis of the figures is summarized in Table 2.

Table 1. *The distribution of cyst measurements*

Mean cyst diameter (μ)	Number of cysts			
	Strain A in HSre	Strain M in HSre	Strain A in hs	Strain M in hs
9·5	6	0	0	0
10·5	50	1	5	0
11·5	102	7	67	2
12·5	212	43	165	15
13·5	242	126	270	44
14·5	201	193	241	106
15·5	119	192	155	188
16·5	43	194	75	190
17·5	13	139	15	200
18·5	9	62	6	118
19·5	2	28	0	61
20·5	1	11	0	46
21·5	0	3	0	16
22·5	0	1	1	7
23·5	0	0	0	3
24·5	0	0	0	4

Table 2. *Statistical constants of cyst measurements*

Source of cysts	Mean diameter of 1000 cysts (μ)	S.D.* (μ)	Difference between means	S.E.† of difference between means
Strain A in HSre	13·594	1·666	2·15	0·078
Strain M in HSre	15·743	1·830		
Strain A in hs	14·061	1·473	2·81	0·079
Strain M in hs	16·876	2·042		
Strain A in HSre	13·594	1·666	0·47	0·070
Strain A in hs	14·061	1·473		
Strain M in HSre	15·743	1·830	1·13	0·087
Strain M in hs	16·876	2·042		

* S.D. = standard deviation. † S.E. = standard error.

In each pair of groups, the difference between the mean diameters is significant, the standard of significance being taken as a difference between the means of at least three times the standard error.

Although the ranges of the cyst sizes were very similar in the four different cultures, the mean diameters of those formed in cultures of strain M in HSre or hs medium were larger, by approximately 2·5 μ, than those of strain A in the same media, and the statistical analysis supports the view that this difference was not due to chance. In addition, the mean diameters of cysts from cultures of strains

A or M in hs medium were significantly larger than those formed in HSre medium. When the frequency with which the cysts occurred was plotted against the cyst diameters, normal distribution curves were obtained for both strains in each of the media studied (Figs. 1, 2). The dispersion of measurements about the means was greater with the cysts obtained from strain M, in which, consequently, the

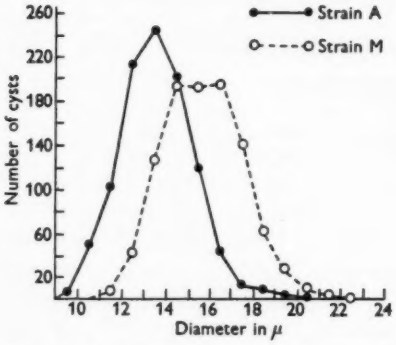

Fig. 1. *E. invadens*. Distribution curves of cyst size of strains A and M grown in HSre medium.

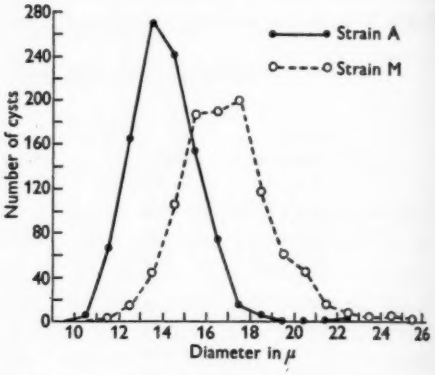

Fig. 2. *E. invadens*. Distribution curves of cyst size of strains A and M grown in hs medium.

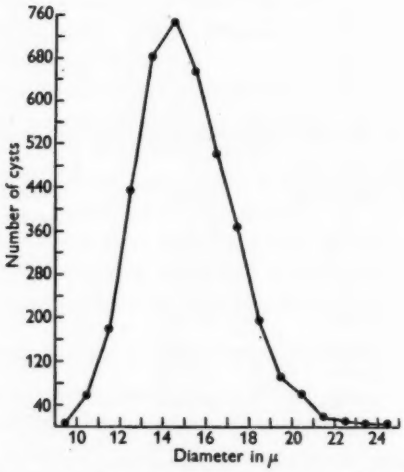

Fig. 3. *E. invadens*. Distribution curve of cyst size of strains A and M grown in HSre and hs media.

standard deviations were larger than in the measurements of cysts from strain A. When the data obtained from the cysts of both strains in HSre and hs media were combined, a unimodal curve was obtained, and the mean diameter of the cysts of both strains considered together was 14·5 μ; in this instance, the mean diameter was identical with the mode (Fig. 3).

DISCUSSION

The unimodal curves obtained from measurements of cysts of strains A and M of *E. invadens* indicate that in each strain only one race, as determined by cyst size, was present. Evidence was obtained, however, that the size of the cysts was influenced by the concomitant bacteria and the media in which they were formed. Thus the cysts of strain A were significantly smaller than those of strain M grown in the same media, and since strain M was derived from strain A, this difference cannot be attributed to racial characteristics, but must be the result of the different bacterial flora of the two strains. Similarly, the smaller size of the cysts of each strain in HSre than in hs medium can be attributed to the influence of the different media.

The influence of the bacterial flora and media on the size of the cysts appears to be correlated with their effect on the growth rates of the cultures in which the cysts were formed. Studies on the numbers of amoebae produced by the two strains in these media (McConnachie, unpublished) have shown that the multiplication rate of strain A is considerably higher in both HSre and hs media than that of strain M; and, in both media, smaller cysts were formed by strain A than by strain M. Similarly, the slightly smaller cysts formed by strain A in HSre than in hs medium were correlated with a higher growth rate in the former medium; although strain M formed smaller cysts in HSre than in hs medium, no detectable difference was found between its rate of growth in the two media. Approximate measurements of the size of the amoebae formed in the two strains indicated that in HSre medium the amoebae of strain A were smaller than those of strain M, and it may be concluded that a high multiplication rate is associated with a decrease in the size of the amoebae, and hence of the cysts formed from them.

Hitherto, the influence of the environment on the size of the cysts of intestinal amoebae has received little attention. Dobell & Jepps (1918) observed that cysts produced in infections of *E. histolytica* which relapsed after unsuccessful emetine treatment, tended to be larger than those produced by the same strain before therapy, and they concluded that the size of the cysts might be modified by a change in the environment, but they did not stress the importance of environmental factors in determining cyst size. Chang (1946) noted that the cysts formed in cultures of *E. histolytica* with low oxidation-reduction potentials and very high rates of multiplication, were a few microns smaller in diameter than those produced in cultures with higher oxidation-reduction potentials and lower multiplication rates; the present findings confirm Chang's observation, which, however, was not supported by any data. Geiman & Ratcliffe (1936) found that the amoebae formed in cultures in *E. invadens* tended to be larger than those formed *in vivo*, and this they attributed to the ingestion of starch by the amoebae maintained *in vitro*; they were unable to explain, however, why this increase in size was maintained by the amoebae and cysts formed in animals experimentally infected with culture cysts.

Wenyon & O'Connor (1917) and Dobell & Jepps (1917, 1918) were the first workers to recognize the existence of strains of *E. histolytica* producing cysts of

different sizes, and they concluded that this species comprised several races or pure lines, each distinguished by the size of the cysts which it produced. Dobell & Jepps (1918) compared the sizes of the cysts passed by different patients infected with *E. histolytica*; curves plotted to show the frequency distribution of cysts from each of four infections were unimodal, while the mode for each was different (6·6, 8·3, 13·3 and 15·0 μ), and it was concluded that this provided proof of the existence of four separate races of *E. histolytica*. From a fifth infection a bimodal curve was obtained, with the modes at 6·6 and 11·6 μ, and it was considered that in this case two races were present, one of which was identical with one of the races already recognized; two more infections were studied which also comprised two races (modes 8·3 and 11·6 μ). Dobell & Jepps claimed to have demonstrated from this data that there were at least five races of *E. histolytica*, and they suggested that even more might be identified. These workers also concluded that since two races may co-exist in the same host, the size of the cysts was not determined by the host, even although, as previously mentioned, they had observed that drug treatment may affect cyst size.

From data obtained from thirty different infections, Malins Smith (1918) concluded that there were only two races of *E. histolytica*, but Dobell (1919) criticized the validity of this conclusion, as Malins Smith measured not more than fifty cysts from any one infection, and obtained a bimodal curve from the pooled results of observations on thirty infections; such treatment of data, however, is surely legitimate if the character under investigation is non-adaptive, and not influenced by the host. Further evidence for the existence of only two races of *E. histolytica* was produced by Gnezdilov (1934) and by Sapero, Hakansson & Louttit (1942), who analysed statistically the pooled measurements of cysts made by themselves, Dobell & Jepps and Malins Smith; in addition, statistical analysis of the data obtained by Dobell & Jepps showed that there was no justification for the conclusions reached by these workers. Sapero and his colleagues state that while there is no evidence that the large race of *E. histolytica* has ever changed into the small race, or vice versa, certain 'observations strongly suggest that environment may play a role in accounting for the minor variations seen in mean diameters of strains belonging to either race'. The present observations provide proof that variations in the size of the cysts of a species of *Entamoeba* may indeed be affected by the environment, apparently as the result of its influence on the growth rate of the amoebae.

The characters distinguishing a race of a species should be stable (i.e. hereditarily fixed), and when such characters are shown to be adaptive, their validity as racial characters is open to question. Cyst size has been shown to be an adaptive character in *E. invadens*, and may be so in other species of *Entamoeba*. The two races of *E. histolytica*, large and small, which have been distinguished on the basis of cyst size, differ also in pathogenicity, and possibly in other characters, and they may therefore be considered to be true races (Sapero *et al.* 1942); within each race, minor variations in size may occur, as the result, for instance, of the different bacterial flora in the hosts in which they are formed, but these variations, as shown by Sapero and his colleagues, always lie within the size range of either race,

each of which has a sharply limited range of cyst sizes. The present measurements have shown that the ranges of cyst sizes of strains A and M of *E. invadens*, which were known to be racially identical, were very similar, but variations occurred in the mean sizes of the cysts. The occurrence of these relatively minor variations has to be taken into account when considering whether differences between two strains are the result of racial characteristics, or different environmental conditions.

Conclusive evidence for the existence of more than one race, on the basis of a difference in cyst size, may be found first, if bi- or multimodal curves are obtained from measurements of the cysts formed in the same host; secondly, if different races retain their distinguishing characters after passage through separate hosts; thirdly, if statistical analysis of measurements from a large number of hosts provides proof of the existence of two or more significantly different groups; or fourthly, if bi- or multimodal curves are obtained from the pooled measurements of cysts obtained from many hosts. Thus Hoare (1940) observed the presence of two races of *E. deblieckí* in a single host, and Neal (1950) found that the sizes of the cysts of two races of *E. muris* were not altered after passage through different hosts, while the work of Sapero and his colleagues illustrates how the methods of statistical analysis may be applied to assess the significance of differences in cyst size.

Examination of the literature describing any single species of *Entamoeba* reveals considerable differences in the sizes of the amoebae and cysts measured by different authors. With regard to *E. invadens*, for instance, Rodhain (1934) gives the range of cyst diameter as 12·75–18·0 μ, Geiman & Ratcliffe (1936) give the range as 11·0–20·2 μ, with an average diameter of 13·88 μ, while Fantham & Porter (1950) found that the size of the cysts of *E. serpentis* (= *E. invadens*) varied in different hosts, and that the cysts formed in six snakes ranged in diameter from 14·7 to 27·4 μ; the present measurements of cysts formed *in vitro* give a mean diameter of 14·5 μ, and a range of 9·0–24·0 μ. Differences in cyst size are frequently used as one of the features by which different species of *Entamoeba* may be distinguished. Thus Geiman & Ratcliffe cite a larger mean diameter of the cysts as one of the differences between *E. invadens* and an *Entamoeba* described by Knowles & Das Gupta (1930); the cysts of these two organisms, however, had a difference in mean diameter of only 2·18 μ, a variation well within the limits observed in the present strains of *E. invadens*. It is suggested, therefore, that differences in the sizes of the amoebae and cysts of intestinal amoebae have only a limited value in distinguishing between species of such organisms, particularly when measurements are made on organisms from a small number of hosts, as it is unlikely that the bacterial flora of different hosts are ever identical.

SUMMARY

In dibacterial cultures of *E. invadens* the size of the cysts was significantly influenced by different species of concomitant bacteria, and by the composition of the media. These factors appear to influence cyst size as a result of their effect on the growth rate of the amoebae; a higher multiplication rate was associated with a decrease in the size of the cysts.

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SOME CESTODES OF THE RAT, *RATTUS RATTUS* LINNAEUS, OF CEYLON AND THEIR EPIDEMIOLOGICAL SIGNIFICANCE FOR MAN

By CHARLES KULASIRI

Department of Parasitology, Medical Research Institute, Colombo, Ceylon

INTRODUCTION

Though *Hymenolepis diminuta* Rudolphi (Hymenolepididae) has long been recognized as a parasite of man in many parts of the world, it was first recorded from this host in Ceylon in 1951. In addition to the specimens reported by de Silva (1951*a*, *b*), a few more have been found during routine examinations in this Institute. These prompted me to investigate the incidence of this parasite in its reservoir host, the rat.

I have also included in this investigation two other cestodes: *H. nana* Dujardin and *Raillietina madagascariensis* Davaine (Davaineidae). It is known that *H. nana* infects man in most parts of the world and that *R. madagascariensis* has been found in man in various tropical and subtropical countries such as Madagascar, Siam, the Philippines, Cuba, British Guiana and Ecuador. Neither of these two species has yet been found in man in Ceylon.

MATERIALS AND METHODS

The rats, all of them subspecies of *Rattus rattus* Linnaeus, were obtained from the rat depot to which rats captured within the municipality of Colombo are brought for destruction. They were kept alive in the laboratory until they were required for examination, but, because the period between capture and examination never exceeded 17 hr., there was no possibility that the infestations found were acquired during captivity.

An external examination of the gut was generally sufficient to reveal the presence of the cestodes. In a freshly killed animal the gut is rather translucent except over the parasites, where an opaque white patch is to be seen. Nevertheless, every gut was opened and carefully examined, except on the very few occasions when there were many more rats than could be disposed of within the time available. In such cases an external examination was used for the preliminary rejection of obviously uninfected guts; all the remainder, including those about which there was the slightest doubt, were subjected to the internal examination. Such examinations were confined to the region between the stomach and caecum, because experience has shown that these parasites are not found outside this region.

The worms were fixed in one of the following fluids, 90% alcohol, Bouin's or Gilson's fixatives, they were stained in acetic acid alum-carmine and mounted for microscopic examination. Although differentiation between the three species with the naked eye was possible with a little experience, all the worms collected were identified after microscopic examination.

RESULTS

These are summarized below:

Total number of rats examined	407
Number infected with *H. diminuta*	27, i.e. 6·63 %
Number infected with *H. nana*	1, i.e. 0·24 %
Number infected with *R. madagascariensis*	12, i.e. 2·95 %

No mixed infections of *Hymenolepis* and *Raillietina* were detected, though two mixed infections of *H. diminuta* and the acanthocephalan *Moniliformis dubius* Meyer, were. It is also of interest that some of the specimens of *H. diminuta* varied in the position of the testes in the manner described by Voge (1952*a*, *b*) and by Schiller (1952) for this species and for other unarmed hymenolepids; and that an irregular alternation of genital pores was found in a few specimens of *R. madagascariensis* in which the arrangement of the genital pores is normally unilateral. It was a similar alternation that led Meggitt in 1927 (see Southwell, 1930) to create the separate and doubtfully valid species *Raillietina fluxa*.

DISCUSSION

Hymenolepis diminuta. The results just quoted show that, of the three parasites, *H. diminuta* was the most frequent in rats within the area of the Colombo municipality. Its high incidence is not to be wondered at, because it is well-established that the rat-to-rat cycle of this species can be completed through a variety of intermediate hosts, including fleas, cockroaches and also many insect pests of stored grain (Chandler, 1949; Craig & Faust, 1945). But it does make necessary some explanation of the extreme rarity of this parasite in man in this same area. De Silva (1951*a*) has suggested that this is mainly due to incorrect determination by laboratory technicians of the egg of this species as that of *Ascaris lumbricoides* Linnaeus. I think this is most unlikely. Clinical manifestations of infection with this parasite are seldom apparent, and detection in the majority of cases from Ceylon was accidental. This being so, the incidence detected in man is likely to be much lower than it actually is, particularly because that section of the population most likely to be parasitized, the poorer classes as a whole, is the very section whose members are not given to seeking medical advice until serious and disabling illness compels them to do so. Of persons seeking medical advice, only those with a history of gastro-intestinal disturbances are given faecal examinations; and these in all dispensaries and in most hospitals in Ceylon are unsatisfactory because the facilities available for such examinations in these places are inadequate. A survey carried out among persons whose occupation included the handling of grain, will probably reveal a much higher incidence than that hitherto recorded.

Hymenolepis nana. One only of the 407 rats examined was infected with *H. nana*, and it is therefore not surprising that no case has yet been reported of this parasite infesting man in Ceylon. Attention must be drawn to the very different conditions prevalent in India (Chandler, 1927), where the parasite is common in man. Accepting the view (Chandler, 1927) that human infections with this species are acquired by contamination from existing murine infections, it will be safe to

predict that *H. nana* will, so long as its rat infestation remains at or near its present low level, continue to be of no importance as a parasite of man in Ceylon.

Raillietina madagascariensis. Much confusion exists regarding the specific identity of the Raillietinas found in man. Lopez-Neyra (1949) recognizes four species only, of which two, *R. madagascariensis* and *R. demerariensis* Daniels are alone relevant to this discussion. These two he differentiates mainly by means of the number of testes and egg-capsules, *R. madagascariensis* having 33–49 testis-follicles and 75–175 egg-capsules, and *R. demerariensis* 50–76 testis-follicles and 150–320 egg-capsules. (Other characters are much more variable and therefore have little diagnostic value.) On this basis I find that the worms described by Cameron & Reesal (1951) and by Stunkard (1953) as *R. demerariensis*, as well as the specimens I have now found in Ceylon rats, all belong to the species *R. madagascariensis* as described by Lopez-Neyra (1949) and by Miyazaki (1950).

The fact that this parasite has not yet been reported from man in Ceylon, although it is moderately frequent in rats here, indicates that the rat-to-man cycle is incomplete. Lopez-Neyra (1949) has suggested that the intermediate hosts may be ants or isopods. But until the life cycle of this species has been fully worked out, it will be premature to speculate upon the reasons for the inability of the parasite to complete the rat-to-man cycle in this country.

SUMMARY

1. *Hymenolepis diminuta* is shown to be quite frequent in the rat, *Rattus rattus*, of Colombo; and reasons are suggested for its apparent rarity in man.

2. An extremely low incidence of *H. nana* in rats was found. It is suggested that this cestode will not be found to parasitize man in Ceylon as long as its murine infection continues to be low.

3. *Raillietina madagascariensis* is not uncommon in rats in Ceylon; but, until its vector has been detected, the possibility that it may infest man in Ceylon cannot profitably be discussed.

4. Instances of variation in the arrangement of genital pores in *R. madagascariensis*, and in the general anatomy of *H. diminuta* were detected.

I am grateful to Dr G. A. R. Perera, Medical Officer of Health, Colombo Municipal Council, for supplying the rats used in this study; to Drs V. Sivalingam and L. G. Jayawardena of this Institute for helpful criticism, and to Dr A. C. J. Weerakoon of the University of Ceylon for revising my manuscript.

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THE AMERICAN SPECIES OF TREMATODE CONFUSED WITH *BUCEPHALUS* (*BUCEPHALOPSIS*) *HAIMEANUS*

By SEWELL H. HOPKINS

Department of Biology, Agricultural and Mechanical College of Texas, College Station, Texas

(With 13 Figures in the Text)

INTRODUCTION

The generic name *Bucephalus* was first used by K. E. von Baer in 1827 for *Bucephalus polymorphus* von Baer, 1827, a cercaria from European freshwater clams. Lacaze-Duthiers (1854) described a similar cercaria, *B. haimeanus* Lacaze-Duthiers, 1854, from the oyster, *Ostrea edulis* L., and the cockle, *Cardium rusticum* in the Mediterranean Sea. The oyster was the type host and Mahon in the Balearic Isles was the type locality. The generic name *Gasterostomum* was proposed by von Siebold in 1848 for the adult forms of what we now know as gasterostomes or bucephalids; von Siebold himself suggested that *Bucephalus polymorphus* von Baer might be a larval stage of *Gasterostomum fimbriatum* von Siebold. Wagener (1858) discussed the probable life cycle of *Bucephalus polymorphus*: the cercaria from fresh-water clams was supposed to encyst on the gills of *Cyprinus* and to develop into mature *Gasterostomum fimbriatum* in perch and pike. This theory was supported by Giard (1874), and Ziegler (1883) considered it proved and concluded that *G. fimbriatum* von Siebold was a synonym of *Bucephalus polymorphus* von Baer. Poche (1907) formally declared *Gasterostomum* a synonym of *Bucephalus*, and set up the family Bucephalidae with *Bucephalus* von Baer, 1827, as type genus.

As *B. polymorphus* (syn. *Gasterostomum fimbriatum*) seems to be the only recognized species of bucephalid in the fresh waters of Europe, there is no reason to doubt the validity of the life cycle which was pieced together by various workers on the basis of the morphological similarities of the stages in clams, cyprinids, and predaceous fishes. However, the attempt to do the same for *Bucephalus haimeanus* has led only to confusion, because there are many species of marine bucephalids in European waters. By the end of the nineteenth century ten species of *Gasterostomum* had been described from adult specimens in European marine fishes, but only one species of marine cercaria had been recognized, all bucephalid cercariae from marine molluscs being identified as *Bucephalopsis haimeanus* (Lacaze-Duthiers, 1854). The subgenus *Bucephalopsis* was named by Diesing (1855) for the cercaria from *Ostrea edulis*, because published descriptions had led Diesing to believe that Lacaze-Duthiers's species had a digestive system very different from that of the fresh-water *Bucephalus polymorphus*. Later European workers found immature gasterostomes encysted in *Gadus morrhua*, *G. minutus*, *G. callarias*, *G. aeglifinus*, *Phycis blennioides*, *Pleuronectes platessa*, *Belone belone*, *Gadus merlangus*, and lampreys. Those in *Gadus* spp. and in lampreys were en-

cysted in nervous tissues, those in *Belone* were encysted in liver, gonads, and peritoneum, and those in the plaice were encysted in muscles. Most authors considered all of these to be immature stages of *Gasterostomum gracilescens*, which becomes mature in *Lophius piscatorius*. Giard (1874) thought that the immature bucephalids which he found encysted in *Belone vulgaris* were later stages of the oyster cercaria, *Bucephalus haimeanus*. Cercariae identified as *B. haimeanus* were reported from *Cardium edule* by Huet (1888), Johnstone (1904, 1905), and others; from *Mactra solida* by Huet (1893); from *Tapes decussatus* and *T. pullastra* by Vaullegard (1894), and from several other marine bivalves by Pelseneer (1906). This brings the European literature up to the time of Tennent.

The first bucephalid reported in American waters was the cercaria from the oyster, which McCrady (1874) described as *Bucephalus cuculus*. McCrady compared his species with the drawings and descriptions of *B. haimeanus* and pointed out several differences. However, Tennent (1905, 1906, 1909) assumed that the cercaria from the American oyster (*Crassostrea virginica*) was the same as the one from the European oyster (*Ostrea edulis*). In fact, Tennent was strongly inclined to believe that all bucephalids, adults and larval forms, fresh-water and marine, were merely 'physiological varieties' of one species. It is this opinion of Tennent's that has caused most of the confusion. Tennent found immature encysted bucephalids (metacercariae) in *Menidia* and adults in *Strongylura*, and assumed that they were the same species as the oyster cercaria. He never obtained any experimental evidence of a connexion between these forms. He did prove that the encysted forms in *Menidia* would excyst, live, and grow when fed to predaceous fishes, but he did not complete these experiments. Tennent's most successful and most important experiment, and the only one which established a connexion between the molluscan and fish stages of a bucephalid, was the one in which he injected faeces of gars, *Lepisosteus osseus*, into the mantle cavities of twenty-six Fort Macon oysters. The gars, caught in Newport River near oyster beds known to be infested with *Bucephalus*, contained numerous adult gasterostomes. Tennent did not describe or draw these gasterostomes because he believed that all gasterostomes belonged to one species. After one month, twenty-two of the twenty-six oysters were still alive, and all of these contained *Bucephalus* sporocysts. Since the oysters came from a bed where many oysters had previously been examined and found free of bucephalids, and since the experimental oysters were kept in a live box in a place where no natural bucephalid infections were found, it seems safe to conclude that Tennent did prove that some unknown gasterostome of *Lepisosteus* was capable of infecting oysters, and of developing in oysters at least to the sporocyst stage. But he did not prove that there was any connexion between the oyster cercaria and the gasterostomes of *Menidia* and *Strongylura*, which were the ones that he drew and described.

Nicoll (1914) undertook a revision of the Bucephalidae. He assumed, on the basis of Tennent's publications and previous statements in the European literature, that *Bucephalus haimeanus*, the cercaria, developed into *Gasterostomum gracilescens*. The subgeneric name *Bucephalopsis* had been assigned by Diesing (1855, 1858*a*) to this cercaria. *Gasterostomum gracilescens*, the supposed adult form

of this species, had a plain muscular sucker without the 'hood' or papillae found in some other bucephalids. Nicoll therefore used *Bucephalopsis* as the generic name for those bucephalids which have 'a simple globular muscular sucker' in the adult stage, with *B. gracilescens* as type species. This would have been a valid procedure if it had been proved that Lacaze-Duthiers's cercaria actually developed into a gasterostome with a plain sucker, but such was not the case. To this day, no life cycle of any European bucephalid has ever been established by experiment. In fact, the only bucephalids whose life cycles have been established are *B. elegans* Woodhead, 1930, *Rhipidocotyle papillosum* (Woodhead, 1929), and *R. septpapillata* Krull, 1934. All of these are American fresh-water species. The cercariae of *Bucephalus elegans* and the cercariae of the two species of *Rhipidocotyle* do not show any signs of the papillae or hoods which distinguish the adults of these species from the adult forms which Nicoll assigned to *Bucephalopsis*. So far, there is no way to tell which genus of the Bucephalidae a given cercaria belongs to, until the life cycle is worked out by means of experimental infections. Lacaze-Duthiers's cercaria from the oyster would be just as likely to develop into a *Bucephalus* or a *Rhipidocotyle* as into one of the species which Nicoll assigned to *Bucephalopsis*.

Therefore, Nicoll was wrong in using *Bucephalopsis* as the name of a genus to include those species whose adults have suckers without hoods or papillae. No connexion between these forms and the subgenus *Bucephalopsis* Diesing has been proved, nor is such a connexion any more probable than a connexion between *Bucephalopsis* Diesing and any other gasterostome genus such as *Bucephalus*, *Rhipidocotyle*, or even *Prosorhynchus*. If the life cycle of *Bucephalus haimeanus* Lacaze-Duthiers is worked out any time in the future, and the adult form is found to belong to a genus other than *Bucephalus*, then the name *Bucephalopsis* Diesing, 1855, is available to replace the name now used for that genus, for *Bucephalopsis* is an older name than *Rhipidocotyle* Diesing, 1858*b*, *Prosorhynchus* Odhner, 1905, or any other generic name (except *Bucephalus*) which has been used for gasterostomes. (See Article 6 of the International Code of Zoological Nomenclature, on the status, of subgeneric names, in *Proc. Biol. Soc. Wash.* **39**, 75–104, 1926.)

According to my interpretation, the genus of gasterostomes with a sucker and without a hood, papillae, or other accessory structures or projections at the anterior end, now has no valid name. I propose the new name *Bucephaloides* for this genus to replace the generic name *Bucephalopsis* Nicoll, 1914, *nec* Diesing, 1855. The type species is *B. gracilescens* (Rudolphi, 1819).

Until more is known about Lacaze-Duthiers's cercaria, the name *Bucephalopsis* Diesing can be used for that species only. Since 1854 there has been no published report, so far as I have been able to determine, of a bucephalid cercaria in *Ostrea edulis*, the type host. The European oyster biologists with whom I have corresponded have never seen such a parasite in an oyster. Numerous findings of bucephalid sporocysts and cercariae in *Cardium edule* and other marine bivalves have been reported from Europe under the name of *Bucephalus haimeanus* or *Bucephalopsis haimeana*, but it is not at all certain that they belong to the same species as the oyster cercaria, and in any case nothing is known of the life cycle of the cockle cercaria either.

AMERICAN BUCEPHALIDS FROM *STRONGYLURA* AND *MENIDIA*

Even before Tennent's investigation, Edwin Linton had found immature bucephalids in *Menidia* and adults in *Strongylura*. It was the bucephalids from these hosts which were most thoroughly studied by Tennent. In his 1940 paper, Linton has reviewed and summarized the trematode studies made during his many years of work at Woods Hole, Massachusetts. In this publication, Linton applied the name *Prosorhynchus gracilescens* (Rudolphi) to all of his bucephalids from *Menidia* and from *Strongylura*. He also placed some specimens from *Menticirrhus* and from *Trachurops* in the same species.

I have studied the bucephalids of silversides and needle gars at Grand Isle, Louisiana, and at Port Aransas, Texas. I have also looked at a few bucephalids from *Strongylura* at Gloucester Point, Virginia. All of the needle gars were tentatively identified as *S. marina*. This identification was confirmed by Dr Gordon Gunter and Mr Henry Hildebrand in the case of the specimens examined at Port Aransas. The silversides examined at Port Aransas were identified as *Menidia beryllina peninsulae* by Dr Gunter. The specific identity of the *Menidia* examined at Grand Isle is not known.

Three distinct species of adult bucephalids were found in the intestines of *Strongylura*. One of these, a new species of *Bucephaloides*, was found only at Port Aransas, where it was very abundant. The two species of *Rhipidocotyle* were found at Grand Isle and in Virginia as well as at Port Aransas. One of these, *Rhipidocotyle transversale* Chandler, 1935, was originally described from immature specimens in *Menidia*, and I have found it in many silversides both at Grand Isle and at Port Aransas. *Rhipidocotyle transversale* in *Menidia* is always encysted in ovoid cysts, which occur in the coelome, liver, fat bodies, mesenteries, and body wall; the most common location is on the outside of the intestinal walls and in the intestinal mesenteries. The *Bucephaloides* found in *Strongylura* was also found very abundantly in *Menidia* at Port Aransas, but was never found encysted; the metacercariae, many of which were as large as adults and differed from them only in the immature form of the uterus and in the absence of eggs, were always found free in the body cavity.

The three species found in *Strongylura marina* are described below.

Rhipidocotyle transversale Chandler, 1935 (Figs. 1–3 and 10*a*)

With the characters of the genus. Length of body 0·8–1·3 mm., width 0·35–0·60 mm.; depth (dorso-ventral dimension) about equal to width when killed by heat in unflattened condition. Anterior sucker 0·18–0·26 mm. long and 0·2–0·30 mm. wide, with a muscular extension or so-called 'hood' projecting dorsally from its anterior edge, without papillae or 'tentacles'. Numerous gland cells lateral, dorsal, and posterior to anterior sucker, with ducts running over and around anterior sucker to open near anterior tip of body. Mouth near junction of first and second thirds of body length, or in middle third but anterior to the middle of the body length. Pharynx nearly spherical, 0·06–0·10 mm. in diameter and in length.

Intestine pouch-shaped, dorsal to pharynx. Vitelline follicles in a single compact group or filling a transverse zone extending across the width of the body just posterior to the cephalic gland cells and just anterior or dorsal to the pharynx and intestine. Ovary nearly spherical, just posterior or lateral to the pharynx and intestine. Uterus coiling posterior to pharynx and intestine and dorsal to testes, mostly on left side of body lateral and posterior to testes. Eggs 20–25 by 10–14 μ;

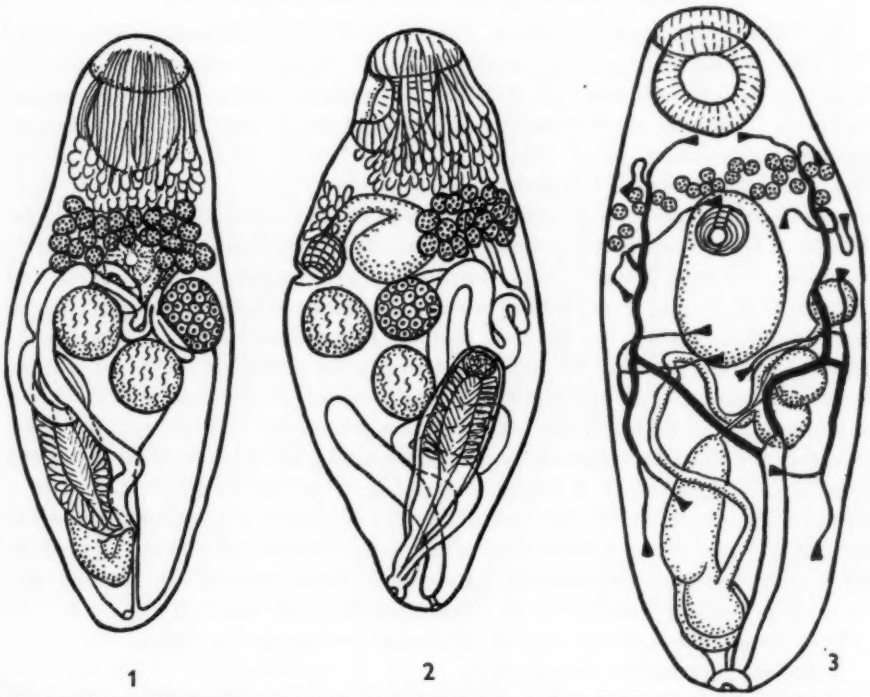


Fig. 1. *Rhipidocotyle transversale* Chandler. Dorsal view of stained and mounted adult from intestine of *Strongylura marina*.

Fig. 2. *Rhipidocotyle transversale* Chandler. Lateral view of stained and mounted adult from intestine of *Strongylura marina*.

Fig. 3. *Rhipidocotyle transversale* Chandler. Dorsal view of living metacercaria from cyst in body cavity of *Menidia* sp., showing the entire excretory system but omitting the cephalic glands.

width one-half to seven-tenths of length. Testes nearly spherical, in ventral half of body just posterior to pharynx and intestine, and either tandem, oblique, or nearly symmetrical. Cirrus pouch extending forward to level of anterior testis, which is approximately in middle of body length. Common genital pore ventral to posterior tip of body. Excretory bladder extending forward to level of posterior testis; main collecting tubes on each side running into anterior end of bladder. In middle of body length, each collecting tube divides, near the lateral margin of the body, into an anterior and a posterior branch. Flame cell formula

$$2\,[(2+2)+(2+2)].$$

FINAL HOST. *Strongylura marina* (Walbaum), needle gar or billfish.

SECOND INTERMEDIATE HOST. *Menidia beryllina* (Cope) and other species of *Menidia*, silversides.

GEOGRAPHICAL DISTRIBUTION. Port Aransas and Galveston Bay, Texas; Grand Isle (Barataria Bay), Louisiana; York River, Virginia; Woods Hole, Massachusetts.

Rhipidocotyle lintoni n.sp. (Figs. 4, 5 and 10*b*)

With the characters of the genus. Length of body 0·45–1·00 mm., width 0·25–0·45 mm., widest part anterior to middle of body. Anterior sucker nearly spherical, 0·15–0·25 by 0·15–0·25 mm.; its diameter is approximately one-fifth the length of the body. There is a small muscular extension of the anterior wall of the sucker, forming a diminutive 'hood'. Compare Linton (1940, p. 31): 'the cap is reduced to a buttonlike process'. Vitelline follicles just posterior to anterior sucker, either in a single transverse zone or in two lateral groups which almost meet in the middle. Mouth in middle or slightly anterior to middle of body length. Pharynx nearly spherical, diameter 0·05–0·07 mm. Intestine pouch-shaped, dorsal or dorsal and anterior to pharynx. Ovary lateral or slightly anterior to level of pharynx. Testes just posterior to pharynx, tandem, oblique, or symmetrical. Cirrus pouch extending forward to level of testes and nearly to middle of body length; its length is approximately one-third of the body length (sometimes more). Uterus extensively coiled both anterior and posterior to pharynx and intestine, filling all the space not filled by other organs and often hiding all other internal structures from sight. Eggs 2·5 to 3 times longer than wide, 25–30 by 8–12 μ, with a conspicuous operculum on one end. Excretory bladder not extending forward to level of posterior testis; main collecting tubes running into anterior end of bladder. Each main excretory tube divides, at the level of the pharynx or forward, into an anterior and a posterior branch. Flame cell formula 2 [(2 + 2) + (2 + 2)].

FINAL HOST. *Strongylura marina* (Walbaum), needle gar or billfish.

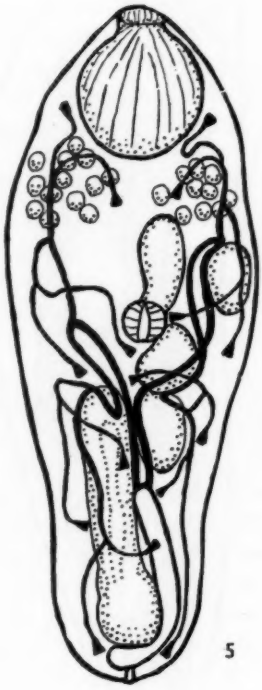
INTERMEDIATE HOSTS. Not known.

GEOGRAPHICAL DISTRIBUTION. Grand Isle, Louisiana; York River, Virginia; Woods Hole, Massachusetts.

Bucephaloides strongylurae n.sp. (Figs. 6, 7 and 10*d*)

With the characters of the genus. Body length approximately 4 times width (length 2·0–2·5 mm., width 0·6–0·8 mm.). Anterior sucker nearly spherical; diameter approximately 0·3–0·4 mm. A conspicuous bunch of gland cells on each side and posterior to anterior sucker, with numerous ducts running dorsally and laterally past anterior sucker and opening near anterior tip of body. Mouth in middle third of body length, anterior to mid-point. Pharynx length approximately half the diameter of anterior sucker. Intestine sack-like, in middle third of body. Vitelline follicles in longitudinal rows or compact bunches lateral to pharynx and intestine. Ovary nearly spherical, to right of median longitudinal plane through body, lateral or posterior to pharynx and intestine. Testes tandem or oblique, posterior to ovary; anterior testis may overlap level of ovary. Anterior end of cirrus pouch at level of testes, more than half-way from posterior tip of body to

pharynx. Posterior end of cirrus pouch opening into a common genital atrium, which opens on ventral side of posterior tip of body. Uterus convoluted, mostly on left side of body between levels of vitellaria and cirrus pouch and on right side



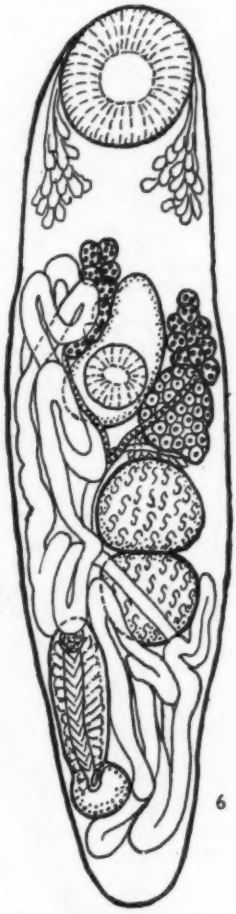


Fig. 4. *Rhipidocotyle lintoni* Hopkins. Dorsal view of stained and mounted adult from intestine of *Strongylura marina*.

Fig. 5. *Rhipidocotyle lintoni* Hopkins. Dorsal view of living adult from intestine of *Strongylura marina*, showing the entire excretory system but omitting the uterus and eggs.

Fig. 6. *Bucephaloides strongylurae* Hopkins. Dorsal view of stained and mounted adult from intestine of *Strongylura marina*.

posterior to posterior testis. Eggs 20–22 by 12–14 μ (alive in water). Excretory bladder extending forward only to level of anterior testis, where the right and left collecting tubes run into the anterior end of the bladder. Each main collecting tube divides, lateral to intestine, into anterior and posterior branches. Flame cell formula 2 $[(4+4+4)+(4+2+2)]$, or close to this formula.

FINAL HOST. *Strongylura marina* (Walbaum), needle gar or billfish.

SECOND INTERMEDIATE HOST. *Menidia beryllina* (Cope, Gulf silversides. Metacercariae not encysted, but free in body cavity.

TYPE LOCALITY. Port Aransas, Texas.

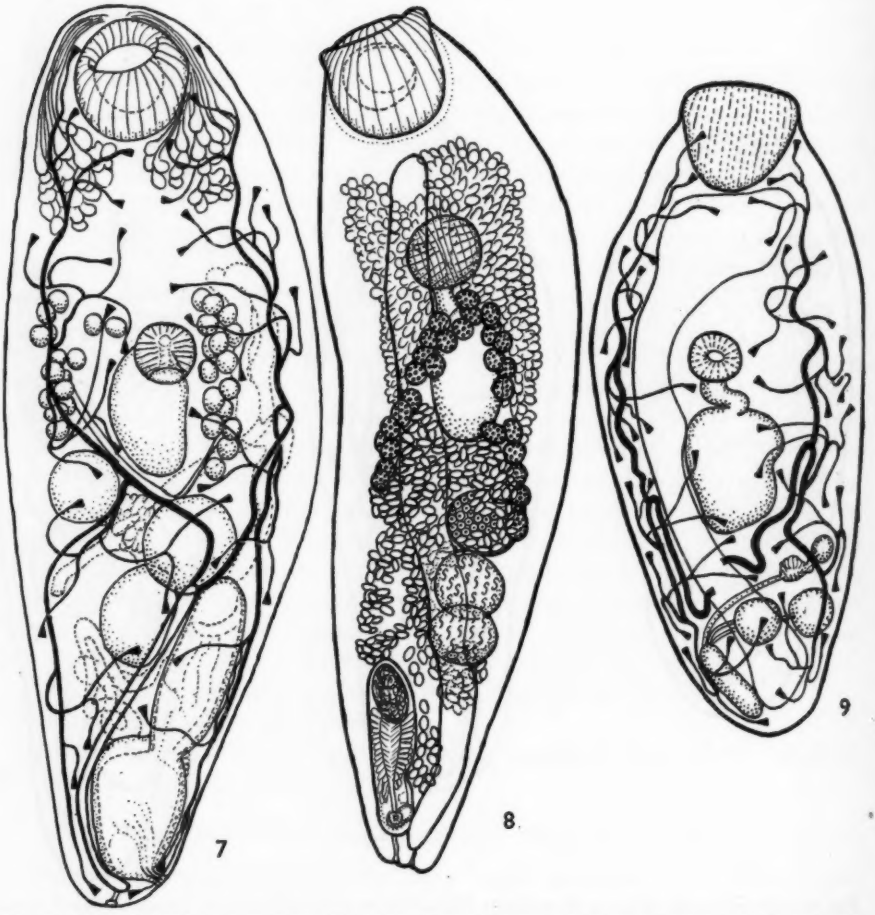


Fig. 7. *Bucephaloides strongylurae* Hopkins. Ventral view of living metacercaria from body cavity of *Menidia beryllina*, showing entire excretory system.

Fig. 8. *Rhipidocotyle lepisostei* Hopkins. Dorsal view of stained and mounted adult from intestine of *Lepisosteus spatula*.

Fig. 9. *Rhipidocotyle lepisostei* Hopkins. Dorsal view of living metacercaria from cyst in fin ray of *Mugil cephalus*, showing entire excretory system but omitting gland cells.

AMERICAN BUCEPHALIDS FROM *LEPISOSTEUS* AND *MUGIL*

As noted above, Tennent never did establish any connexion between the oyster cercaria and the bucephalids of *Menidia* and *Strongylura*. His only successful experiment involving both fish and oyster stages was the one in which he infected

oysters by injecting faeces of *Lepisosteus osseus*. The mature gasterostomes in the gars were not drawn or described; Tennent simply assumed that they were the same species as the ones in *Strongylura marina*.

No gasterostomes have been reported from any species of *Lepisosteus* from Tennent's time until now. Working at Grand Isle, Louisiana, in 1950 and 1952, I found and studied adult bucephalids from the intestine of alligator gars, *L. spatula* Lacépède, from Barataria Bay. A single species was found, and it was present in all of the four gars examined. It is very different from all of the three species found in *Strongylura marina*. It seems probable, though of course not certain, that this is the species which Tennent used in his oyster infection experiment. The new species from the alligator gar is described below. Immature specimens (encysted metacercariae) of what is almost certainly the same species were found in young mullets (*Mugil cephalus* and *M. curema*).

Rhipidocotyle lepisostei n.sp. (Figs. 8, 9 and 10*c*)

With the characters of the genus. Body length 1·2–2·0 mm.; width at widest point (level of pharynx) about 0·3–0·6 mm., approximately one-third of length in unflattened specimens killed by heat. Anterior sucker nearly spherical, or hemispherical with the anterior edge flattened, 0·16–0·26 by 0·16–0·30 mm. A weakly developed 'hood' forms a slight ridge which runs transversely across the anterior edge of the sucker and sometimes shows ear-like lobes or projecting corners at its lateral extremities. There are no papillae or tentacles. Cephalic glands, if present, are very inconspicuous, but there are two large, conspicuous masses of gland cells massed around each side of the pharynx and intestine, with ducts running toward the mouth. In mature specimens the mouth is in the anterior third of the body; immature specimens have the mouth farther back, at or near the middle of the body. The large pharynx measures 0·10–0·15 by 0·10–0·16 mm. The intestine is pouch-shaped and posterior to the pharynx, to which it is joined by a short oesophagus. The vitelline follicles form an inverted U or V posterior to the pharynx and anterior to the testes. The spherical ovary is about half-way from the pharynx to the posterior end, on the right side. The testes are close behind the ovary and are tandem, oblique, or symmetrical in position. The relatively small cirrus pouch, only about one-fifth as long as the body, extends forward to the level of the posterior testis. The coils of the uterus fill the region posterior to the mass of glands around the digestive system. The eggs are 27 to 35 μ long and 15 to 21 μ broad; the width averages six-tenths of the length. The excretory bladder runs forward to or almost to the anterior sucker; its anterior end is sometimes pressed against the anterior sucker in such a way that it appears to be Y-shaped, but actually it is undivided as in all other bucephalids. The main excretory vessels run into the excretory bladder on each side at about the level of the ovary or half-way between the

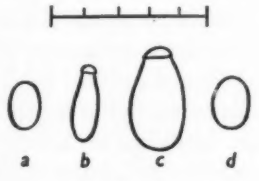


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Fig. 10. Eggs of the four species, drawn to the same scale (scale line = 50μ): *a*, *Rhipidocotyle transversale*. *b*, *R. lintoni*. *c*, *R. lepisostei*. *d*, *Bucephaloides strongylurae*. All from balsam mounts.

pharynx and the posterior end. The main vessels divide, lateral to the intestine, to form anterior and posterior branches. The flame cell formula approximates 2[(4+4+4)+(4+4+4)].

FINAL HOST. *Lepisosteus spatula* Lacépède, the alligator gar.

SECOND INTERMEDIATE HOST. *Mugil cephalus* L., striped mullet, and *M. curema* Cuv. & Val., white mullet. In very small mullets the encysted metacercariae are in the gill region or in the body cavity; in larger mullets most of the encysted metacercariae are inside of the rays of the fins, especially the caudal fin; very large mullets usually do not contain any metacercariae.

GEOGRAPHICAL DISTRIBUTION. Barataria Bay, Louisiana.

AMERICAN BUCEPHALIDS IN OYSTERS

The first bucephalid reported from North America was *Bucephalus cuculus* McCrady, 1874; the sporocysts and cercariae of this species were found in four oysters, *Crassostrea virginica* (Gmelin), at Charleston, South Carolina, in 1868. McCrady's description and figures are inadequate for identification of the species, by modern standards, but McCrady did compare his species with the figures and descriptions in European publications, including Lacaze-Duthiers's original account, and pointed out at least three differences between his species and *Bucephalus haimeanus*. Bucephalid sporocysts and cercariae were found in New Jersey oysters by Julius Nelson (1890, 1903, 1915); Nelson reported that approximately 1% of the oysters in New Jersey were infected by this parasite.

Tennent (1905, 1906, 1909) unfortunately disregarded McCrady's name for the American oyster bucephalid and called it *B. haimeanus* Lacaze-Duthiers. He found an incidence of 5–6% infection in oysters from Rappahannock River (Virginia) and 23–25% infection in oysters collected near Beaufort, North Carolina. In some beds, as high as 33% of the oysters contained *Bucephalus* sporocysts and cercariae. However, no infection could be found in oysters from the highly saline waters near Fort Macon, which provided Tennent with a stock of uninfected oysters for use in experiments.

B. cuculus (under that name) was reported from Louisiana oysters by Glaser (1904), and later by other workers at the Gulf Biologic Station, Cameron, Louisiana.

Since Tennent's time, very little work has been done on the American oyster bucephalid. A. E. Woodhead of Michigan has studied it, but his findings have never been published. Oyster biologists have contributed some information, mostly in the form of incidental remarks in reports on other phases of oyster biology. *B. cuculus* is now known to occur in *Crassostrea virginica* from New Jersey to Texas, but its distribution is not continuous throughout this range; there are many places where the parasite is absent or at least extremely rare. The incidence of infection is highest in low salinities and in the smaller, more landlocked, or more inland estuarine waters, and is lowest in the large bays and sounds where conditions approach truly marine or oceanic conditions. The host, *C. virginica*, is itself an estuarine species adapted to the variable temperature, high turbidity, and low salinity of river mouths, and *Bucephalus cuculus* is largely confined to the

least saline parts of its host's range. On the other hand, *Ostrea edulis* is a truly marine species which lives in offshore beds; it does flourish in the lower portions of estuaries, but it can not stand as much dilution of sea water as *Crassostrea virginica*. *Bucephalus haimeanus* was originally found by Lacaze-Duthiers in oysters growing in the highly saline Mediterranean Sea. Even if the cercaria in

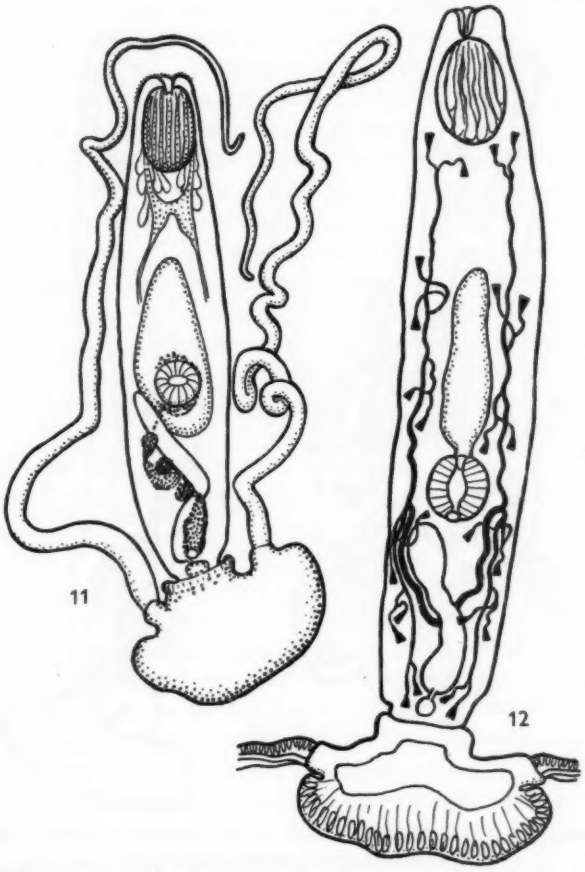

Fig. 11. *Bucephalus cuculus* McCrady, cercaria from *Crassostrea virginica*; ventral view of stained and mounted specimen.

Fig. 12. *Bucephalus cuculus* McCrady, cercaria from *Crassostrea virginica*; ventral view of living specimen, showing entire excretory system but omitting gland cells (except those in the tail).

the more euryhaline cockle is the same as the one in the European oyster, the distribution of *B. haimeanus* is much more oceanic and less estuarine than that of *B. cuculus*. Even if there were no morphological differences between the American and the European cercariae, it would be very difficult to believe that two forms with such striking ecological and physiological differences could belong to one species.

All authors have assumed that there is only one species of bucephalid in

American oysters. If this is true, the correct name of the species must be *B. cuculus* McCrady, 1874. There is no theoretical reason why two or more species could not occur in *Crassostrea virginica*, but so far there is no evidence of specific differences between the oyster cercariae described by different American workers. We can only call all bucephalids from our oyster *Bucephalus cuculus* until specific differences can be demonstrated.

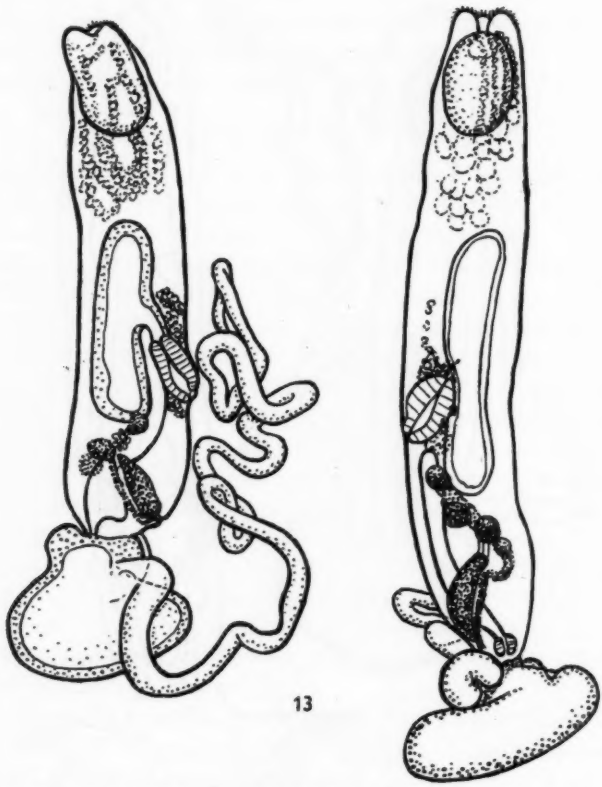

Fig. 13. *Bucephalus cuculus* McCrady, cercaria from *Crassostrea virginica*; lateral views of two stained and mounted specimens.

B. cuculus cercariae from Louisiana oysters are shown in Figs. 11–13. Note that the main excretory tubes do not open into the anterior end of the excretory bladder, but empty into each side of the bladder near the middle of its length. This alone would be sufficient to exclude the possibility that this cercaria could develop into any of the three species found in *Strongylura marina*. The flame cell pattern of the oyster cercaria seems to be 2 [(2+2+2)+(2+2+2)], as shown in Fig. 13. However, it is very difficult to see all parts of the excretory system at once in this cercaria, and I am not absolutely certain that there may not be some flame cells which I have not seen. The excretory bladder extends forward only to the level of the pharynx, which is slightly posterior to the middle of the body. The structure

at the anterior end of the body could be interpreted as either a sucker or a rhynchus, according to preference. Gland cells, not very distinct or conspicuous in life, occur both around the anterior sucker (or rhynchus) and around the pharynx. The genital anlagen are all posterior to the pharynx and intestine. Cutaneous gland cells, scattered over the entire body surface, are rather conspicuous in life and help to obscure the details of internal structures such as the excretory system. The tail stem sometimes has a slight tendency toward a bilobed shape, but is never distinctly divided into two parts as in some other species. Its shape varies according to the salinity of the water, becoming more swollen and globular in low salinity and becoming smaller and more distinctly bilobed in high salinity. Gland cells which secrete a sticky substance are located along the posterior edge of the tail stem and in the furci. The furci are attached to the ventral surface of the anterior border of the tail stem, as shown in the figures. The cercariae from the oysters have never been seen in the act of swimming, and it is not certain that they are able to swim. Oysters have been seen to eject small swarms of cercariae, and it seems possible that cercariae thus forcibly ejected might become entangled in the gills or fins of fishes (by means of their sticky, thread-like tail furci) and thus be able to penetrate the second intermediate host without ever swimming.

Measurements of heat-killed specimens in water, unflattened, are as follows: Length of body 0·24–0·40 mm. (0·30–0·35 in most specimens, and usually about 4 times width). Width of body 0·05–0·12 mm. Length of tail stem 0·04–0·10 mm. Width of tail stem 0·07–0·17 mm. (1·5 to 3 times length). Length of anterior sucker 43–75 μ. Width of anterior sucker 28–55 μ. Diameter of pharynx 26–29 μ.

DISCUSSION

The descriptions and figures published by Linton and Tennent indicate that at least two species of *Rhipidocotyle*, *R. transversale* and *R. lintoni*, were seen by them. The account of '*Prosorhynchus gracilescens*' given by Linton (1940) definitely includes both of these species. Linton's figure 246 was evidently drawn from a specimen of *Rhipidocotyle transversale*, while his figures 245 and 248 seem to represent *R. lintoni*; specimens of the latter species were aptly described by Linton (pages 31 and 32) as having the 'cap reduced to a button-like process'. It is not certain that the specimens from *Menticirrhus* belong to *Rhipidocotyle lintoni*, but this seems probable from Linton's account.

The drawing of the adult '*Gasterostomum gracilescens*' from *Strongylura marina* in Tennent (1906), which Eckmann (1932) mentioned as the best representation of the species characteristics of *Bucephalopsis haimeana*, may have been made from a specimen of *Rhipidocotyle lintoni*; it certainly resembles this species very closely, and does not resemble any other species which I have found in *Strongylura marina*. Points of resemblance of Tennent's figure and *Rhipidocotyle lintoni* include: (1) the shape of the body, (2) the relatively large size and the large aperture of the anterior sucker, (3) the anterior projection of the anterior sucker (in Fig. 46), suggesting the presence of the small 'hood' or 'buttonlike process', (4) the position of the vitellaria, close behind the sucker, (5) the position of the mouth, pharynx, and intestine, (6) the position of the ovary, just anterior to the pharynx, (7) the

position of the testes, close to ovary and pharynx (however, Tennent shows the testes farther apart than is usual in *R. lintoni*), and (8) the large cirrus pouch extending forward to the level of the testes. It is certain that at least some of Tennent's specimens belonged to the genus *Rhipidocotyle*, for one of his drawings (Fig. 54 in Tennent's 1906 paper) shows a section through an anterior sucker with a 'hood'.

Tennent's specimens from *Strongylura marina* probably included both *Rhipidocotyle transversale* and *R. lintoni*, and his encysted bucephalids from *Menidia* were probably *Rhipidocotyle transversale*. It is possible that he also saw *Bucephaloides strongylurae* in *Strongylura* or *Menidia*, or both. However, *B. strongylurae* has not been found at Grand Isle and none of the Atlantic bucephalids described or illustrated by Linton and others seems to resemble this species very closely, so it seems more probable that *B. strongylurae* does not occur east of the Texas coast.

B. strongylurae is the only species of the genus *Bucephaloides* (= *Bucephalopsis* Nicoll *nec* Diesing) now known to occur in *Strongylura marina*. Even if Tennent did see this species, he did not obtain any evidence that it was connected with the oyster cercaria, for he did not conduct any experiments which would connect the oyster cercaria with stages in either *Menidia* or *Strongylura*. *Bucephaloides strongylurae* differs from *B. gracilescens* (Rudolphi, 1819) by the relatively larger size of its anterior sucker (in relation to the pharynx and to the body length), and by the fact that its vitelline follicles and excretory bladder do not extend forward as far as they do in the type species. *B. gracilescens* adults occur in the intestine of the fishing frog or angler, *Lophius piscatorius* L., which is a cold-water and deep-water bottom fish. According to European reports, the encysted metacercariae of *Bucephaloides gracilescens* are found in the nervous tissues of members of the Gadidae or cod family, which are also cold water and deep-water fishes. It may be assumed, therefore, that the first intermediate host is a mollusc which lives in cold waters. *B. strongylurae*, on the other hand, is found in *Strongylura marina* and *Menidia beryllina* in warm, shallow, inshore waters. Incidentally, Linton (1940) does not mention ever having found a bucephalid in *Lophius piscatorius*, although he and his associates examined many of these fish over a period of many years. It seems likely that *Bucephaloides gracilescens* does not occur in American waters, probably because the molluscan host is lacking.

As noted above, Tennent never produced any evidence that any of the species in *Strongylura* or *Menidia* had any connexion with the oyster cercaria, *Bucephalus cuculus*. Tennent (1909) did succeed in infecting oysters by injecting eggs of a bucephalid from *Lepisosteus osseus*. He did not describe or picture this bucephalid. No bucephalid from a *Lepisosteus* has ever been described, prior to the present paper, and there seems to be no mention of one in the literature other than Tennent's report. The new species *Rhipidocotyle lepisostei*, from *Lepisosteus spatula*, is very distinct from all of the species in *Strongylura* and *Menidia*. It is probable, though not certain, that the bucephalids Tennent found in *Lepisosteus osseus* were *Rhipidocotyle lepisostei*. I have found the metacercariae of *R. lepisostei* in many mullets, *Mugil cephalus* and *M. curema*, but never in *Menidia*.

Circumstances did not permit me to spend more than a few days at a time in

a marine laboratory, and therefore I was unable to complete any infection experiments. Comparative study of excretory systems shows that *Bucephalus cuculus* has no connexion with any of the three bucephalids in *Strongylura marina* (*Rhipidocotyle transversale*, *R. lintoni*, and *Bucephaloides strongylurae*), for all of them have the main collecting tubes emptying into the anterior end of the excretory bladder, while the collecting tubes of the cercaria open into the sides of the bladder near the middle of its length (as Tennent also observed). Besides, *Rhipidocotyle transversale* and *R. lintoni* have only eight flame cells on each side of the body, have the 2 [(2+2)+(2+2)] flame cell formula, while the oyster cercaria has at least twelve flame cells on each side.

R. lepidostei has the collecting tubes opening into the sides of the bladder as in the oyster cercaria. Both the metacercariae in *Mugil* and the adults in *Lepisosteus* have more flame cells than the cercaria, but it seems possible that additional flame cells might be produced during the development of the metacercaria from the cercaria (as is known to occur in some trematodes). Incidentally, two other bucephalids found at Grand Isle, a *Bucephalus* and a *Bucephaloides* from *Cynoscion nebulosus*, also have the tubes opening into the side of the bladder posterior to the pharynx. However, both of these species have the 2 [(2+2)+(2+2)] flame cell formula, which eliminates any possibility of a connexion with the oyster cercaria.

If future investigations should establish by experiment that the oyster cercaria, *Bucephalus cuculus*, develops into the *Rhipidocotyle lepisostei* metacercaria in mullets, or that eggs of *R. lepisostei* from gars are infective to oysters and develop into sporocysts which produce *Bucephalus cuculus* cercariae, then the name of the species would become *Rhipidocotyle cuculus* (McCrady, 1874), and *R. lepisostei* would be a synonym. However, this still remains to be proved. I do not know of any other trematode life cycle which involves both marine (oyster and mullet) and fresh-water (gar) hosts, but this combination does not seem improbable in estuaries where such species normally live together.

SUMMARY

1. The bucephalid cercaria from *Crassostrea virginica* (Gmelin), the commercial oyster of the Atlantic and Gulf coasts of North America, has been called *Bucephalus haimeanus* Lacaze-Duthiers and *Bucephalopsis haimeana* (Lacaze-Duthiers). Neither of these names is correct. *Bucephalus haimeanus* Lacaze-Duthiers, 1854, is a parasite of the European oyster, *Ostrea edulis* L. (and possibly of cockles, *Cardium* spp.) in oceanic or near-oceanic habitats, while the American species is a parasite of an estuarine oyster and is found almost entirely in the least saline parts of its host's range. The correct name of the American oyster bucephalid is *Bucephalus cuculus* McCrady, 1874.

2. The life cycles of all European marine bucephalids are still unknown. The life cycles described in the European literature are based entirely on morphological resemblances which are not close enough to be convincing, and none of them has been tested by experiment. Bucephalid cercariae do not show any of the features which are used to distinguish genera. So far as present evidence goes, *Bucephalopsis haimeana* is just as likely to develop into a *Rhipidocotyle*, a *Bucephalus*, or a

Prosorhynchus as it is to develop into a member of the genus *Bucephalopsis* Nicoll, 1914, *nec* Diesing, 1855.

3. *Bucephalopsis* Diesing, 1855, is the name proposed by Diesing for a subgenus created especially for the cercaria *Bucephalus haimeanus* Lacaze-Duthiers, 1854. Nicoll had no right to use this name for the genus of gasterostomes which, as adults, have a muscular sucker at the anterior end and do not have accessory structures such as a hood or papillae. Up to the present, there is still no evidence that this genus has any connexion with Lacaze-Duthiers's cercaria, other than common membership in the family Bucephalidae. Therefore the new name *Bucephaloides* Hopkins has been proposed to replace the generic name *Bucephalopsis* Nicoll, 1914, *nec* Diesing, 1855, with *Bucephaloides gracilescens* (Rudolphi, 1819) as the type species. This genus does not include *Bucephalus haimeanus* Lacaze-Duthiers because the metacercaria and the adult form of that species remain unknown.

4. Tennent (1905, 1906, 1909) did not prove that there was any connexion between the oyster cercaria, *Bucephalus cuculus* McCrady, and the immature bucephalids in *Menidia* or the adult bucephalids in *Strongylura*. His drawings show that at least some of the bucephalids which Tennent studied had a hood and therefore belong in *Rhipidocotyle*. Re-examination of the bucephalids in *Strongylura marina* reveals that at least three species of bucephalids occur as adults in that host; these are described in this paper as *Rhipidocotyle transversale* Chandler, 1935, *R. lintoni* Hopkins, and *Bucephaloides strongylurae* Hopkins. *Rhipidocotyle transversale* and *Bucephaloides strongylurae* were also found in an immature (metacercaria) stage in *Menidia*, and were the only bucephalids found in *Menidia* during this study. The excretory systems of the three species in *Strongylura* all have features which exclude the possibility that they could develop from the oyster cercaria, *Bucephalus cuculus*.

5. Tennent's (1906) drawing (fig. 46) of an adult bucephalid from *Strongylura marina*, which Eckmann (1932) mentions as the best representation of the species characteristics of *Bucephalopsis haimeana* (Lacaze-Duthiers), was probably made from a specimen of *Rhipidocotyle lintoni*.

6. Tennent (1909) proved that eggs or larvae from an unknown adult bucephalid in *Lepisosteus osseus* could infect oysters, *Crassostrea virginica*, and develop into sporocysts which would grow in oysters for at least one month. This is the only experimental proof of a connexion between an adult bucephalid in a fish and a larval bucephalid in an oyster. Until the present, no bucephalid from a gar (*Lepisosteus*) had ever been described. In the present paper *Rhipidocotyle lepisostei* Hopkins is described from adults in *Lepisosteus spatula*, the alligator gar, in Louisiana. The metacercariae of *Rhipidocotyle lepisostei* are abundant in the fin rays of mullets, *Mugil cephalus* and *M. curema*. The excretory system of this species is not identical with that of *Bucephalus cuculus*, but is not so different as to exclude the possibility that the oyster cercaria might develop into *R. lepisostei*. If, in the future, *R. lepisostei* is proved to be the adult form of *Bucephalus cuculus*, the name of the species will become *Rhipidocotyle cuculus* (McCrady), and *R. lepisostei* will become a synonym.

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CHONIOSPHAERA INDICA, A COPEPOD PARASITIC ON THE CRAB *NEPTUNUS* SP.

By C. P. GNANAMUTHU

Director, Zoology Laboratory, University of Madras, S. India

(With 29 Figures in the Text)

The choniostomatids parasitic on crustacea have been exhaustively studied by Hansen. To the six genera and forty-three species he had described in 1897, he added a new genus and three more species in 1904, and seven more species in 1923. Scott (1904, 1905, 1907) described seven more species, Fraenkel (1915) three new species, Monod (1930) one, and Blake (1929) two. In 1929, however, Connolly described a new genus *Choniosphaera* and its type species *C. cancrorum*, from parasites found on American crabs *Cancer amoenas*, *C. irroratus* and *C. borealis*. In the present paper a new species *Choniosphaera indica*, parasitic on the edible crab *Neptunus sanguinolentus* of Madras, is described.

The author is grateful to Mr S. Krishnaswamy, M.Sc., of this department, for drawing his attention to the occurrence of this choniostomatid and for collecting a large number of specimens of it.

OCCURRENCE AND HABITAT

More than 200 ovigerous females, six juveniles, twenty larvae and three males were collected from about six berried crabs. Some of the larvae were obtained from the gills of the hosts, and the rest were collected from their egg masses. When the 'berry' of the crab was teased up in a watch-glass, the larvae separated more easily, while the adults and the immature parasites were found clinging to individual eggs. The cup-shaped mouth-tube is firmly applied to the outer membrane of the host's egg, while the second antennae, the second maxillae and the maxillipedes are used for clinging to the egg. Since the egg appeared undamaged, it is presumed that the parasite sucks the fluids in the egg through a small puncture made and kept open by the needle-like mandibles. In the gills, the larvae were found between the lamellae, probably feeding on the tissue fluids. When they are separated from the eggs, the parasites die in a few hours.

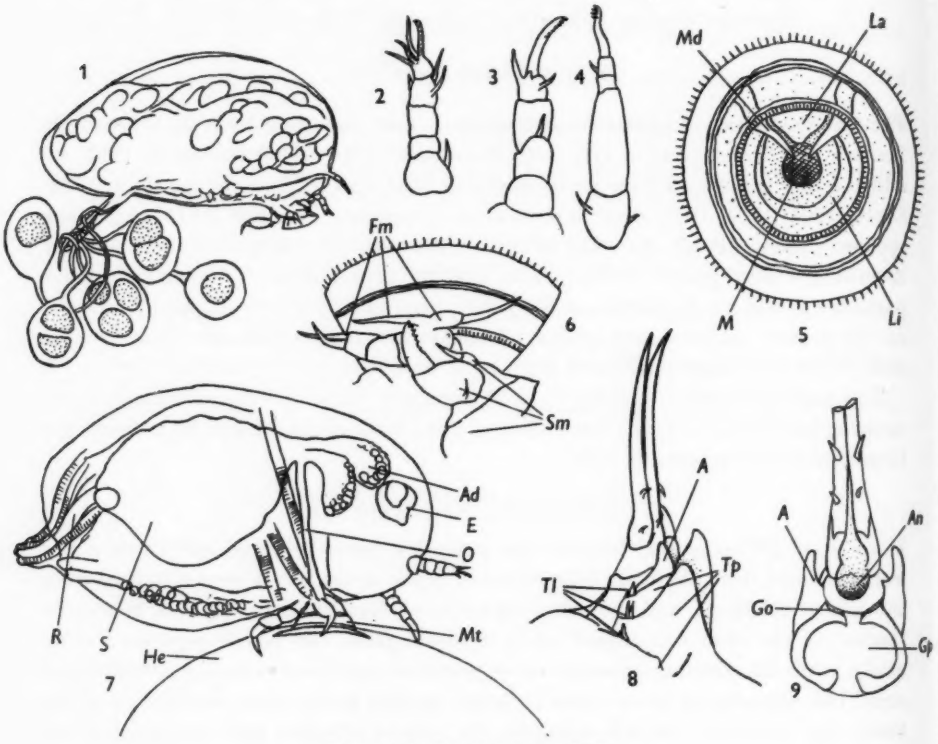
The female

The adult female is seed-like and ellipsoid in outline, and the posterior part of the trunk projects behind the caudal styles. In older forms the trunk extends in front of the antennae and increases in diameter, so that the parasite appears more spherical than before. The body has lost all its segmentation, the abdomen having fused with the cephalothorax. The surface of the body is smooth and devoid of hairs. The parasites are saffron-yellow in colour and mimic the crab's eggs. There is no trace of the eye. The body is opaque.

Size. Egg-bearing adults, measured from the anterior to the posterior margin,

vary in size from 0·8 to 1·2 mm. in length. They are half as wide as long, while the older adults are shorter and wider. The parasite appears to grow in bulk throughout its life.

Appendages. The first antennae are far apart, ventral and subterminal. They have five articles. The first joint bears a spine, the fourth has three spines, and the

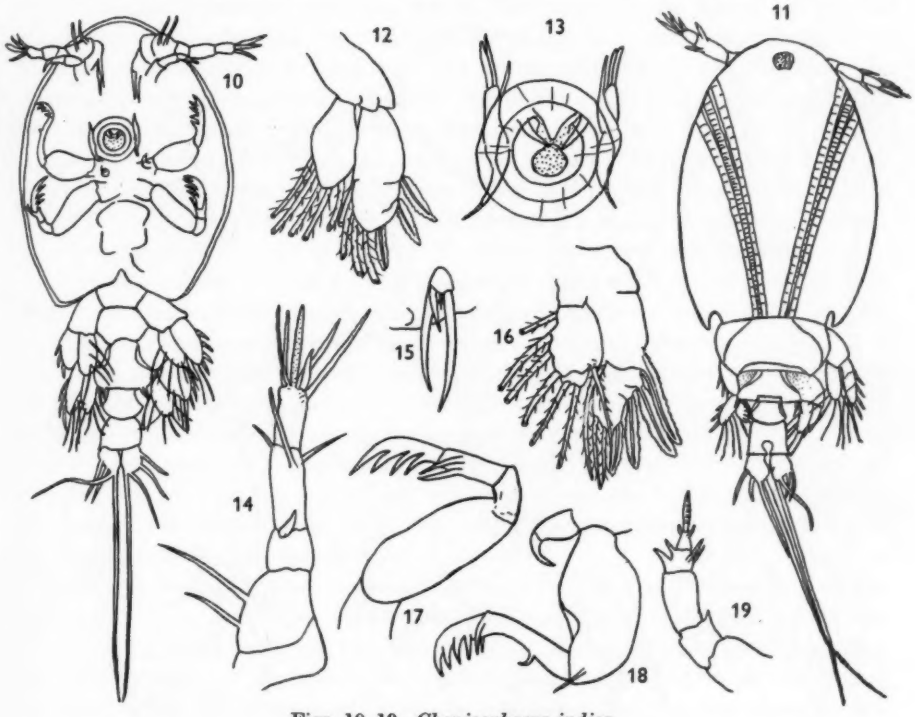


Figs. 1–9. *Choniosphaera indica*

Fig. 1. Young ovigerous female, × 100. Fig. 2. First antenna, × 600. Fig. 3. Second antenna, × 600. Fig. 4. Maxillipede, × 600. Fig. 5. Oral view of the mouth-tube, × 750. *La*, labrum; *Li*, labium; *Md*, mandible; *M*, mouth. Fig. 6. Side view of the mouth-tube and the maxillae, × 600. *Fm*, first maxilla; *Sm*, second maxilla. Fig. 7. Pupa of the female, × 200. *Ad*, anterior diverticula, *E*, eye; *He*, host egg; *Mt*, mouth-tube; *O*, oesophagus; *R*, rectum; *S*, stomach. Fig. 8. Caudal styles and vestigial trunk legs, × 600. *A*, abdominal plate; *Tp*, three thoracic plates; *Tl*, thoracic legs. Fig. 9. Genital area and bases of the caudal styles, × 750. *A*, abdominal plate; *An*, anus. *Gp*, genital plate; *Go*, genital opening.

terminal joint carries three spines and a blade-like aesthetask. The second antennae are more ventral and are five-jointed. The third joint has a spine, and the terminal joint carries two long slender spines, a short spur and a long stout seta with pectinate teeth at the tip. The mouth-tube occurs at the apex of a prominence on the ventral side of the body and is surrounded by the maxillae and the maxillipedes. It is cup-like, the mouth being at the bottom of the cup. The rim of the cup is perfectly circular and bears a membranous lip. The base of the cup is a firm

circular plate which is not continuous on the anterior side. Fitting into this gap is a triangular labrum with the apex directed towards the central mouth. As Hansen has suggested, the mouth-tube or rostrum is mainly formed by the labium. The mandibles are pointed blades projecting into the bottom of the mouth-tube from in front, one on either side of the triangular labral flap. They are visible only in an oral view. The first maxilla is biramous and is found closely pressed to the



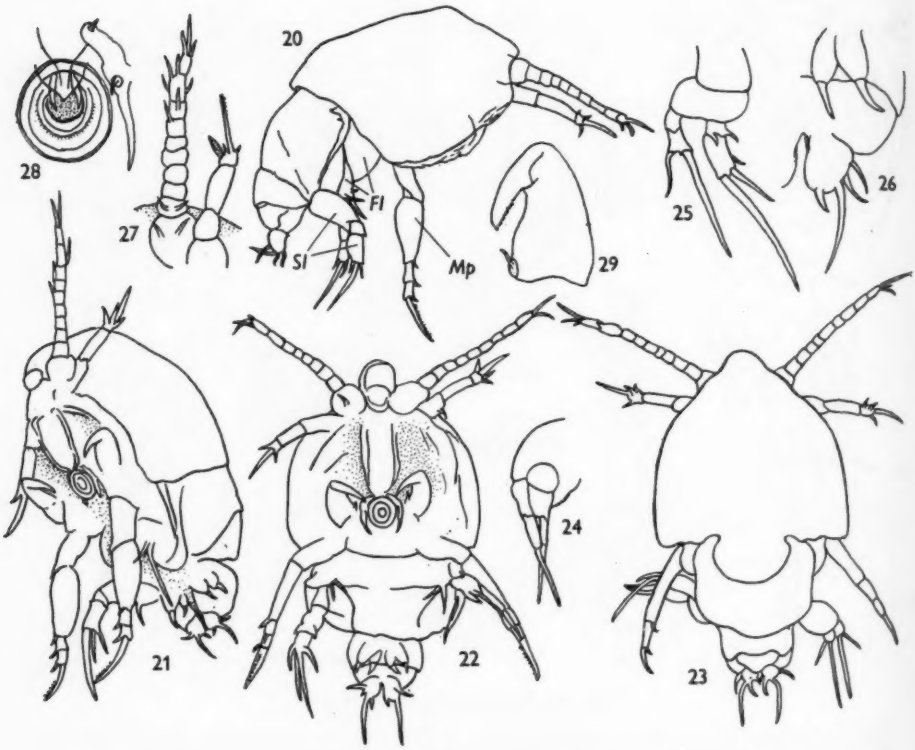
Figs. 10–19. *Choniosphaera indica*

Fig. 10. Ventral view of the copepodite larva, × 200. Fig. 11. Dorsal view of the copepodite larva. Fig. 12. Second thoracic leg, × 600. Fig. 13. Mouth-tube, mandible and first maxillae, × 600. Fig. 14. First antenna, × 600. Fig. 15. Third thoracic leg, × 600. Fig. 16. First thoracic leg, × 600. Fig. 17. Maxillipede, × 600. Fig. 18. Second maxilla, × 600. Fig. 19. Second antenna, × 600.

outer side of the mouth-tube. The inner ramus has two joints, the proximal one bearing a spine directed forwards and the distal one a spine directed backwards and upwards. The outer ramus is three-jointed and is directed forwards and ends in two spines. The second maxilla has a distinct two-articled protopodite bearing two rami. The outer ramus is stout and two-jointed and has a short spine on the proximal joint and a number of stubby teeth on the tapering part of the distal joint. The inner ramus is single-jointed and carries a broad spine. The maxillipede is a long appendage of five unequal joints; it has two spines on its basal joint, a small recurved spine on its fourth joint and it ends in a tuft of pectinate teeth. An inner ramus of the maxillipede was not visible.

The legs of the trunk are reduced to three plates closely arranged near the bases of the caudal styles and bear three pairs of spines. These three plates probably represent the last three segments of the thorax.

Genital area. In front of the base of the caudal styles there is a mid-ventral, elliptical genital plate which bears the genital apertures. The openings of the vesiculae seminales are not distinct in all the specimens. The anus lies at the base of



Figs. 20–29. *Choniosphaera indica*

Fig. 20. Dorsolateral view of the male, × 200. *Fl*, first leg; *Mp*, maxillipede; *Sl*, second leg. Fig. 21. Ventrolateral view of the male. Fig. 22. Ventral view of the male. Fig. 23. Dorsal view of the male. Fig. 24. First thoracic leg, × 600. Fig. 25. Second thoracic leg, × 300. Fig. 26. Third thoracic leg and anal lamina of left side, × 600. Fig. 27. First and second antenna, × 300. Fig. 28. Mouth-tube and first maxilla of left side, × 600. Fig. 29. Second maxilla. × 600.

the caudal style behind the posterior border of the genital area. A foliaceous plate on either side of the anus indicates the abdomen, which is fused with the thorax.

Caudal styles. These are two long, stout spines borne on cushion-like bases. The pronounced development of the caudal styles in the adult, and their absence from the immature pupae, is probably related to the fact that the ovisacs are frequently attached by their threads round these styles.

The ovisacs are spherical and are attached by transparent stalks to the genital apertures. As more sacs are formed, the long stalks become entwined in the caudal styles. As many as thirty egg-sacs were found in one female. Each sac is transparent and may have a maximum of three developing embryos. As the parasite becomes surrounded by the crab's eggs, the stalks of its egg-sacs become entangled by the crab's eggs and, when the parasite moves voluntarily or by accident, the egg-sacs become detached from the parent. They appear paler, whiter and smaller than the crab's eggs. As many as a hundred egg-sacs, derived probably from several parasites, may be seen together among the crab's eggs. Because the parasite grows far bigger than a single egg of the crab, it is probable that the parasite feeds, during its life, on more than one egg.

The immature female. Hansen (1897) gave the name 'pupae' to the immature females. More than eight of them were collected from the egg-mass of a single crab. They are much smaller than the adults and they lack the caudal styles. They varied in length from 0·27 to 0·8 mm. The different parts of the alimentary canal were visible, because the body was transparent. The oesophagus meeting the large bag-like stomach at right angles, the pair of glandular diverticula opening into the stomach and the numerous bundles of muscles extending from the oval region to the dorsal side of the body recall similar features of Lernaeids. The eye is large and pigmented red and blue.

The copepodite larva. The ovisacs examined showed embryos with faint indications of rudiments of a fourth pair of appendages, but it was impossible to say whether the larva, when it hatched, would be a metanauplius or a copepodite. In spite of very careful search, no free nauplius was encountered, but Connolly (1929) has described the nauplius of *C. cancrorum.*

Twenty copepodites were collected from the gills and egg-masses of different crabs and these were alike in structure and size. They were transparent and possessed a reddish median eye. Their powers of swimming were feeble and they died within an hour of removal from host. Because some of them were found attached to the egg of the host, it can be concluded that they are parasitic and cannot live freely.

Size. Excluding the anal laminae, the larvae measured 0·34 mm. in length and 0·16 mm. in breadth across the carapace.

The body. The carapace is about 0·2 mm. long and oval in outline. It is strongly convex dorsally and the pleural edges curve downwards. There are two free thoracic segments. The first is as broad as the posterior margin of the carapace and the second is nearly half its size. Hansen (1897) does not definitely state that the second segment is thoracic, and Connolly (1929) has added it to the abdomen. In this species, however, it is clearly thoracic, because it bears vestiges of the third pair of trunk legs. The abdomen consists of two segments, the anterior one being short and narrow and the posterior one nearly twice as large. The anal laminae are broad, rhomboid plates, each of which bears four long setae. Of these setae one is more than half the length of the entire larva.

Appendages. The first antenna is five-jointed. Terminally it has five setae and one aesthetask. The next segment bears three spines and the third segment one.

The two basal segments are broad, the more distal one bearing two long spines which project far beyond the frontal margin. The second antenna also has five joints. The second joint has a broad tooth-like spine, the third is the longest, the fourth bears four spines and the fifth bears two short spines as well as a long stout seta bearing four fine teeth. The mandible, the mouth-tube and the first maxilla are like those of the adult. The second maxilla is biramous and is better developed than the second maxilla of the adult is. The endopod has three joints; of which the proximal one is very large and stout and bears a spine, the second is narrow and long and carries a spine in the middle and two spines at the distal edge, and the terminal joint bears four large teeth which give it a pectinate appearance. The outer ramus is short and bears a large recurved spine. The maxillipede is uniramous and has five articles. Its terminal joint bears four pectinate spines and the penultimate joint carries two long spines directed forwards.

The first two pairs of trunk legs are natatory, the third pair being vestigial. The first leg has a two-articled protopod bearing two rami. Its exopod is indistinctly three-jointed and bears four leaf-like spines on its outer margin and three long plumose setae on its inner margin. The endopod is faintly divided into two segments and bears seven plumose setae. The second leg is also biramous. Its exopod is faintly marked into three joints which bear three laminate spines on the outer margin and four plumose setae on the inner margin. The endopod is broad and carries five plumose setae distally and one proximally. The third pair of legs are papillae bearing two long spines at the base and two shorter ones at the tip. Connolly (1929) mentions the presence of a long spine on each side of the first abdominal segment and it is probable that this segment really belongs to the thorax and that this spine is the vestige of the third leg.

The male

Three males were collected together with egg-bearing females and copepodites from the egg-masses of crabs. They were alike, but differed in size and structure from the copepodites, the female pupae and the adult females. They showed the marked dimorphism noted by Hansen (1897) in all the species that this author described. The male retains the body-segmentation and appendages characteristic of the larva, but the appendages have lost the natatory form.

Size. Excluding the anal laminae, the body was 0·25 mm. long and 0·14 mm. wide across the carapace.

Body. Although it is shorter than the larva, the male is stouter and more robust. The body is naked and free of hairs. The frontal area is marked by a prominent median lobe, which projects forwards between the bases of the antennae. The submedian skeleton is better developed than it is in the female and the mouth-tube and appendages appear to have moved to the posterior region of the cephalothorax, with the consequence that the maxillipedes project backwards from the hind margin and the first thoracic segment, which is fused with the cephalothorax in all copepods, is more distinct. Dorsally, however, the carapace has a large lobe which covers this segment medially. There are two free thoracic segments, the abdomen being produced to two very narrow segments which are nearly hidden in a dorsal

view. The anal laminae are broad, as those of the larvae are, and they bear four spines, of which one is far longer than the others.

Appendages. The first antennae have ten segments. Hansen (1897) has noted that first antennae of the males of other species of choniostomatidae also have a larger number of segments than the first antennae of the larvae and females have. The terminal segment of the first antenna of the male bears two spines and an aesthetask, the next segment bears four spines, the two basal segments have two spines each and the remaining segments bear one spine each. The second antenna has four articles. The third segment forms half the length of the appendage and the distal joint carries two short spines, a long-toothed seta and a broad aesthetask. The mouth-tube, mandible and the first maxillae are like those of the larva and female. The median skeleton is conspicuous. The second maxilla has two joints, the proximal joint being large and the distal joint forming a finely toothed claw. A small one-jointed spine at the base represents the endopod. The maxillipedes are the largest appendages of the male. They have five articles and taper towards the tip. The distal two segments are short and narrow. There is a short spine on the distal segments, the last of which bears a long terminal spine, toothed at the tip.

Trunk legs. The first pair of thoracic legs, well developed in the copepodite, is reduced in the male. It is biramous, each ramus having two joints and each ramus ending in a long spine. The second pair of thoracic legs is better developed than the first and third pairs are. The protopod has two joints, as the rami also have. Both rami carry two long terminal spines, but the exopod bears three lateral spines as well. The third pair of legs is more or less vestigial, being found pressed close to the second free thoracic segment. Both the exopod and the endopod have one article only which bears a single spine.

No pupa of this male was found. Hansen (1897) states that the larvae of the genus *Sphaeronella* give rise to the adult male without an intermediate pupal stage, and it is probable that the larvae of *Choniosphaera indica* also do this. Such an assumption would account for the increase of the antennal segments from five in the larva to ten in the adult and also for the differences between the form of the maxillae, the maxillipedes, and the trunk legs of the larva and the adult.

TAXONOMIC REMARKS

From the descriptions given by Hansen (1897) of the seven genera parasitic on crustacea other than crabs, it will be obvious that the parasite of the crab described above does not belong to any of Hansen's species. Its lack of an abdomen, possession of caudal styles and the features of its genital area place it nearest to species of the genus *Homoesceles*; but it is differentiated from these by its possession of well-developed antenna and by the vestigial condition of its trunk legs, which are located posteriorly. In these, as well as other features, it resembles the genus *Choniosphaera* established by Connolly (1929), the type species of which *C. cancrorum* is a parasite of crab's eggs. Although Connolly was not able to find the male and the pupa of the female, and although the present author was not able to obtain

the nauplii, the resemblances between the adult female and the larva of *C. cancrorum* and the species described above, justify the inclusion of the species here described in the genus *Choniosphaera*. The differences noticed in the trunk legs, the caudal styles, the maxillipedes, the antennae and the form of the adult female, help to distinguish the present species from *C. cancrorum* Connolly. It is therefore described as a new species, *C. indica*.

Locality. Madras. S. India.

Host. *Neptunus sanguinolentus* Herbst.

Type. Both the holotype (♀) and the allotype (♂) will be deposited in the Indian Museum, Calcutta.

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LICHOMOLGIDES CUANENSIS N.G., N.SP., AN ASCIDICOLOUS COPEPOD OCCURRING IN *TRIDIDEMNUM TENERUM* (VERRILL)

By R. V. GOTTO

Zoology Department, Queen's University, Belfast

(With 20 Figures in the Text)

The copepod described in this paper has been found in Strangford Lough, Co. Down, an area which has already proved rich in ascidicolous copepods of the suborder Notodelphyoida (Gotto, 1952). The present species, however, belongs to the section Poecilostoma of the suborder Cyclopoida, many members of which are associated with marine animals of several different groups. The extremes of retrogressive specialization which characterize many of the notodelphyoids are not, on the whole, so apparent in these cyclopoids, their associative habits often being inferred from general structure rather than proved occurrence with any particular host. Thus a fair number of the types described have been found unattached in dredged material, the possible host being any one of several species taken in the same haul. Associations of an apparently loose nature are therefore sufficiently frequent to render the present form of particular interest since it has so far been found exclusively in the compound ascidian *Tridemnum tenerum* (Verrill), in which it is localized in the extensive cloacal cavities. Further search may of course reveal alternative hosts, since the family Lichomolgidae, to which it undoubtedly belongs, has a wide range of acceptable hosts. Indeed the genus *Lichomolgus* is known to occur in association with sponges, polychaets, nudibranchs, gasteropods, cephalopods, holothurians and ascidians—and one species, *L. agilis* (Leydig) has been recorded from a score of different nudibranchs.

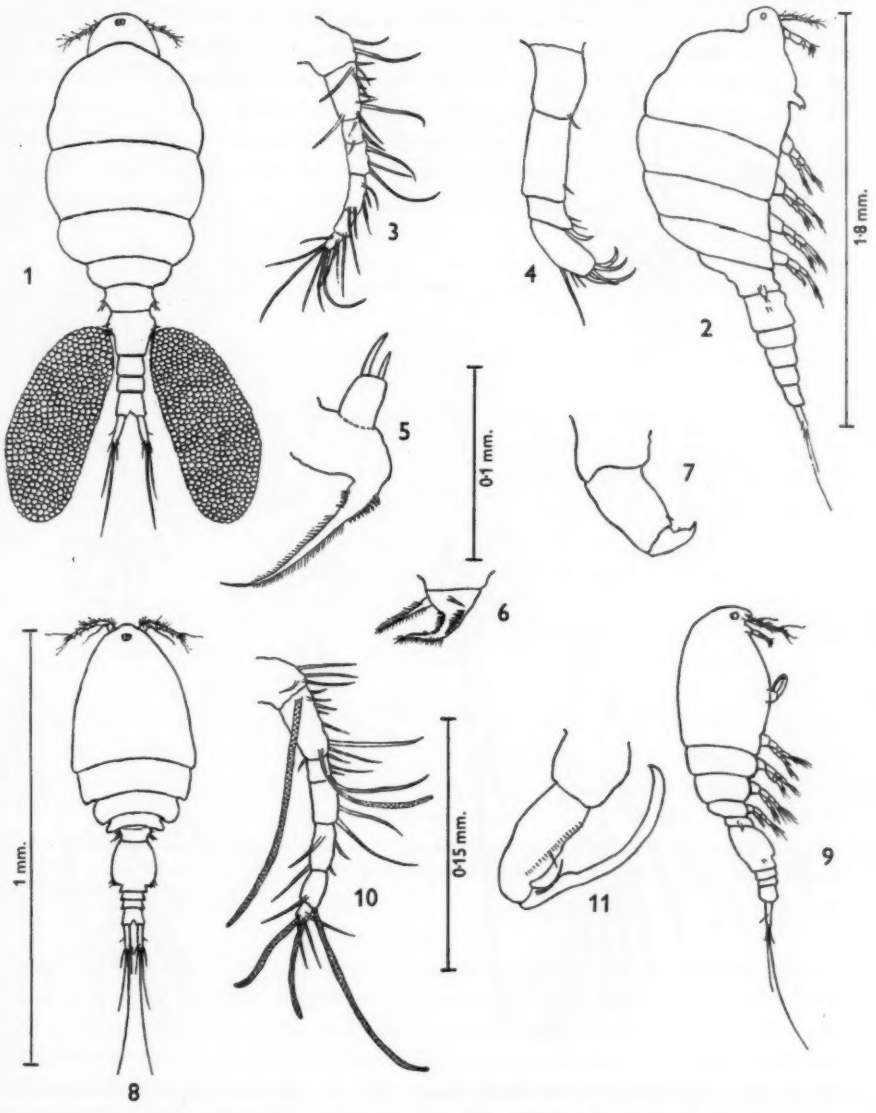
The structural characteristics of the Strangford specimens appear to the present author sufficiently novel to warrant the erection of a new genus and species, for which the name *Lichomolgides cuanensis* is hereby proposed. With regard to the naming of the mouthparts in the description which follows, the terminology first proposed by Hansen (1893) has been adopted. Although realizing that the terms used by Sars (1918) have the sanction of familiarity, it seems best to accept the now widely held view that only one pair of maxillipeds occurs in the Copepoda. As to the much-debated question of the presence or absence of a mandible in the poecilostomes, the present author has followed the example of Sars (1918), and this appendage is accordingly regarded as missing. The arguments in support both of Hansen's terminology and the absence of a mandible in the Poecilostoma have been cogently presented by Lang in a recent paper (1946).

Lichomolgides cuanensis n.g., n.sp.

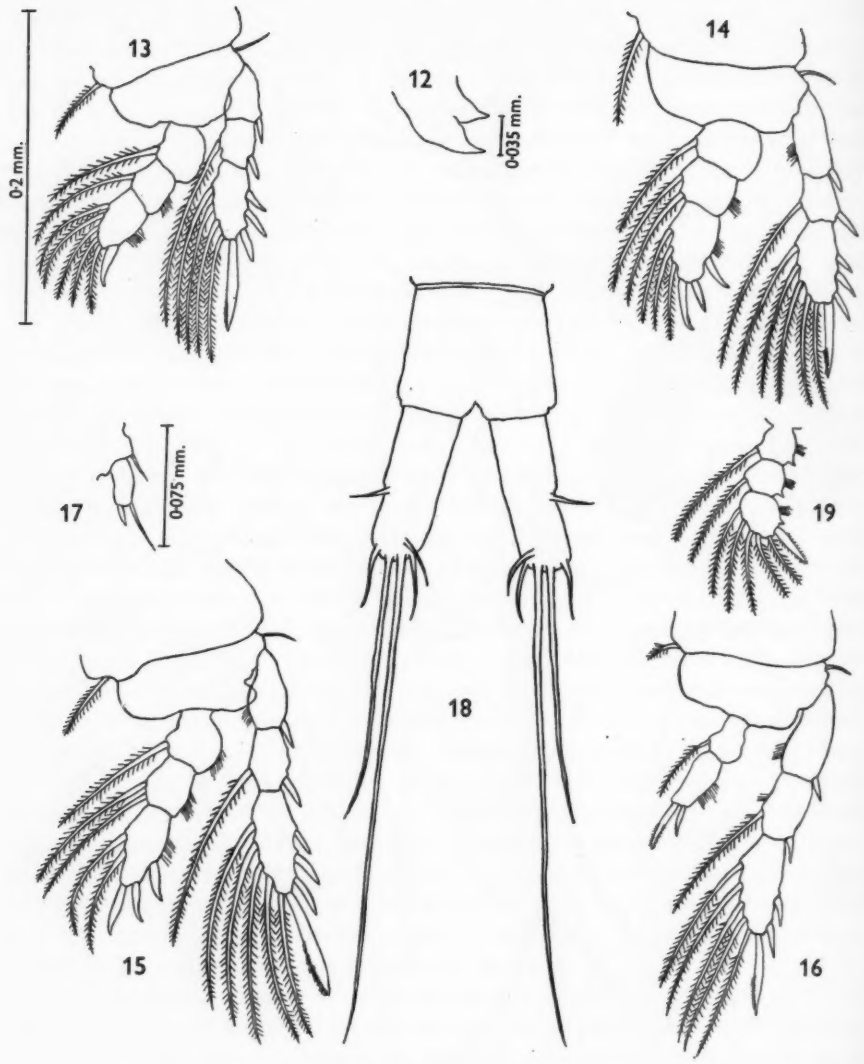
Description of females (Figs. 1, 2). Size of adult 1·5–2·1 mm. in length from the anterior end to the caudal rami inclusive, with an average of about 1·8 mm. To this the furcal setae add approximately 0·33 mm. The greatest breadth is attained

in the second thoracic segment and is about 0·58 mm. Colour greyish, semi-transparent, with the ovarial tubes pale blue-mauve; eye red. In shape, the thoracic region is strongly inflated dorsally, with the cephalic area sharply delimited from the rest of the cephalothorax. A small rostrum is present. Abdominal region slender with the swollen genital segment slightly exceeding the combined lengths of the two succeeding segments. Two small setae are found dorso-laterally on each side of the genital segment. Furcal rami rather long and slender (Fig. 18), slightly exceeding in length the anal segment. Each bears four non-ciliated setae terminally, of which the second median is the longest, one short subterminal median seta, and one outer seta attached almost at the middle of the ramus. Antennule (Fig. 3) of seven segments, the second being the longest, and moderately setose throughout. The antenna (Fig. 4) consists of four segments, of which the second is the largest. The first and second antennal segments each bear a single short seta distally, while the small third segment possesses two such setae and a curved claw. The last segment carries a fairly long slender seta, two very small setae, and, at the tip, four curved claws. Maxillule (Fig. 5) with the terminal lappet long, slender and densely ciliated almost to the tip; the base slightly swollen and armed with spinules. Palp in the form of a small lobe bearing two stout setae of unequal size. Maxilla (Fig. 6) of two segments, the second being produced into an apical lash armed proximally with spinules and distally with very small fine hairs. A large densely ciliated seta and a small plain seta occur at the base of this joint. Maxilliped (Fig. 7) of three segments, short, stout, and almost devoid of setae, with the distal segment produced into a small claw. Margin of upper lip smooth, and with only a shallow median cleft. From the immediate post-oral region (and probably representing a development of the lower lip) a ventrally produced median structure is apparent, which terminates distally in two slightly recurved hooks lying in the vertical plane, with their points directed posteriorly (Figs. 2, 12). First to third pairs of thoracic limbs (Figs. 13–15) well developed, exopodites and endopodites three-jointed and of nearly equal size, with an extensive armature of spines and ciliated setae. Fourth pair (Fig. 16) with the endopodite of two joints, the distal being provided with two unequal terminal spines. Fifth pair (Fig. 17) very small, the single free joint bearing at its tip a short spine and a longer seta. Egg-sacs elongate-oval, about 1 mm. in length and pale blue-mauve in colour.

Description of male (Figs. 8, 9). Size of adult about 0·74 mm. in length, with the furcal setae adding 0·30 mm. Greatest breadth (at posterior end of cephalothorax) 0·26 mm. Colour almost transparent, with the alimentary canal showing as a yellow-brown streak, and with occasional red flecks; eye cherry-red. Shape typically cyclopoid, with the thoracic region considerably less inflated than in the female. A small rostrum is present. Abdomen about half the length of the thorax, with the genital segment longer than the three following segments combined. The swollen portion of this segment bears two small setae postero-laterally on each side. Furcal rami long, with four terminal setae, one subterminal and one lateral seta on each. Antennule (Fig. 10) very similar to that of the female, but provided with aesthetascs (stippled in figure) on the second, sixth and seventh segments. Antenna, maxillule and maxilla very closely resembling those of the female (Fig. 20).



Figs. 1–11. *Lichomolgides cuanensis* n.g., n.sp. Fig. 1. Dorsal view of ovigerous female. Fig. 2. Lateral view of female. Fig. 3. Female antennule. Fig. 4. Female antenna. Fig. 5. Female maxillule. Fig. 6. Female maxilla. Fig. 7. Female maxilliped. Fig. 8. Dorsal view of male. Fig. 9. Lateral view of male. Fig. 10. Male antennule. Fig. 11. Male maxilliped. The scales are applicable to those structures to which they are immediately adjacent.



Figs. 12–19. *Lichomolgides cuanensis* n.g., n.sp. Fig. 12. Distal portion of post-oral hooked organ. Fig. 13. First leg of female. Fig. 14. Second leg of female. Fig. 15. Third leg of female. Fig. 16. Fourth leg of female. Fig. 17. Fifth leg of female. Fig. 18. Anal segment and caudal rami of female. Fig. 19. Endopodite of first leg of male. The scales are applicable to those structures to which they are immediately adjacent.

Maxilliped (Fig. 11) powerfully developed into a strong grasping organ, with the dactylus long, curved and bearing a single seta at the base. The middle joint possesses a row of delicate spinules and a single longer seta. The posterior margin of the upper lip is minutely spinous. There is no conspicuous development of the lower lip region. First to fourth pairs of thoracic limbs as in the female, but with a small sharp projection at the outer distal end of each endopodite joint (Fig. 19). Fifth pair as in the female. Length of spent spermatophore (as determined from those found attached to the genital segment of the female), 0·1 mm.

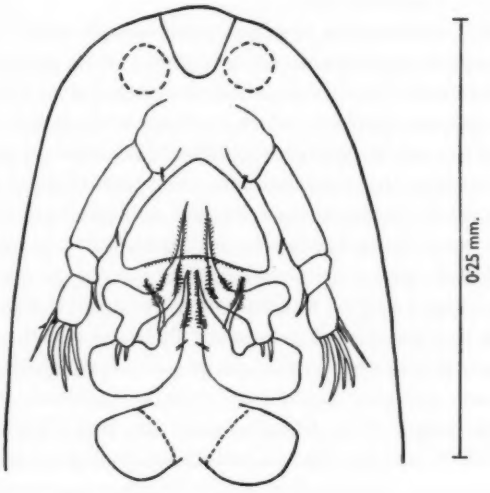


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Fig. 20. *Lichomolgides cuanensis* n.g., n.sp. Ventral view of cephalic area of male, omitting antennules (their bases indicated as circles of dots), and the greater part of the maxillipeds. Semi-diagrammatic.

Bionomics. This copepod is of frequent occurrence in *Trididemnum tenerum* in Strangford Lough, though it appears to be largely confined to those colonies in which spicule development is relatively slight. Host colonies of this type show a high percentage of infection. *Lichomolgides* was first obtained in April 1950, but specimens have since been found over the greater part of the year. Ovigerous females have been noted in April, May, August and October. Males are also fairly abundant, having been seen in February, August, October and December. During October two females were observed with about ten spermatophores adhering to the genital segment of each. This would indicate multiple mating with, probably, a high percentage of successfully fertilized eggs.

Numerous individuals are frequently found in a single host colony. Thus, in May, one colony of about 13 cm.^{2} contained seven female specimens, while in October seventeen females (mostly ovigerous) occurred in a colony of some 40 cm.^{2}. As well

as the commonplace members of the faunal microcosm usually associated with compound ascidians—turbellarians, syllids, bryozoans, amphipods, etc.—another ascidicolous copepod, *Haplostoma banyulensis* (Brément), often occurs with *Lichomolgides* in *Trididemnum*. Considering the comparative lack of depth in didemnid colonies, their tendency to spicule formation in the matrix and the small size of their zooids, it is perhaps remarkable that they should prove so attractive as hosts to copepods. This point was remarked upon by Chatton & Brément (1915), and is well illustrated by Harant's list (1931) in which, out of fifty-two ascidicolous copepods, seven (or over 13%) occur in didemnids. To this number can now be added the cyclopoid described here.

Within its host, *Lichomolgides* occupies what are, in effect, probably the only regions with dimensions practicable for a copepod of its size and shape, viz. the spacious cloacal cavities. In this situation it is assured of a constant stream of water rich in the organic débris on which it presumably feeds. For the most part, adult females *in situ* remain passive until disturbed, when a series of short spasmodic movements carry them further into the cloacal labyrinth. The males are noticeably more active, though neither sex will swim for extended periods in spite of the well-developed thoracic limbs, the females tending to sink passively to the bottom if introduced into a deep vessel. The weight of the large egg-sacs in ovigerous specimens may well be a factor contributing to their sluggish behaviour. In connexion with this question of movement, it is possible that the peculiar post-oral 'hooks' described above may assist progression through the cloacal system by acting as temporary 'anchors' against the exhalant current.

The considerable length of the furcal setae in this genus conflicts to some extent with Sars's view (1918, p. 151), that normal development of these setae in certain lichomolgids indicates an ectoparasitic habit. On the other hand, it may be argued that the association between *L. cuanensis* and *Trididemnum* might be of such comparatively recent origin that regressive specialization is as yet incomplete.

Since obvious aggregations of cyclopoid nauplii have not so far been found in the host, it can be assumed that this stage represents the distributive phase in the life-cycle, the newly hatched nauplii either swimming or being swept from the cloacal apertures, and ultimately seeking fresh host colonies.

Syntypes of *Lichomolgides cuanensis* n.g., n.sp. have been sent to the British Museum of Natural History.

Generic diagnosis. Female with thoracic region markedly vaulted, the cephalic area being sharply delimited from the rest of the cephalothorax. General structure of head appendages, thoracic limbs and abdominal region as in the genus *Lichomolgus*. In the immediate post-oral area there is a conspicuous ventrally produced median structure, provided distally with two barely recurved hooks, their points directed posteriorly. Male typically cyclopoid, with no sharp definition of the cephalic area, and lacking the above-mentioned 'hooks'. Other characters similar to those of males of the genus *Lichomolgus*.

Affinities. Were only the structure of the head appendages and thoracic limbs to be considered, it would be difficult to place this copepod other than in the genus *Lichomolgus*. The number and arrangement of spines and setae on the limbs are

precisely similar to eight out of the eighteen species of *Lichomolgus* discussed by Sewell (1949) in this connexion. The only difference lies in the absence of the small distal projection on the outer side of each endopodite joint in the female—a feature present in *Lichomolgus* spp., and retained in the male *Lichomolgides*. The cephalic appendages too are typically lichomolgid, though they also resemble in certain minor characters those of the allied genera *Macrochiron* Brady and *Pseudanthessius* Claus. However, the clear demarcation of the head from the rest of the cephalothorax, as well as the development of the ventral 'hooks', clearly indicate that this form should be accorded separate generic status in the subfamily Lichomolginae.

Nature of association between the copepod and the ascidian. The site occupied by *Lichomolgides cuanensis* within its host raises certain questions of some theoretical interest regarding the whole problem of a satisfactory terminology to describe the varying degrees of intimacy in animal associations. It must be admitted that our knowledge of the many described marine partnerships is exceedingly fragmentary in all save a very few cases. This is made clear by the protean interpretation of such terms as 'inquilinism', semi-parasitism', 'commensalism', etc., in the writings of various authors. Caullery (1952), for example, regards many of the ascidicolous copepods as inquilines, thereby implying that they seek shelter within a natural cavity of the host and divert a part of the collected food to their own use. This would be an extremely useful term were it not for the fact that it holds different connotations for the entomologist, who employs inquilinism to denote a *nest-* or *gall-sharing* association amongst insects.

As Baer (1951) has pointed out, the inter-relations between the partners must be accurately known before their association can be adequately classified. As a contribution towards the necessarily finer definitions of such associations (at least in the ascidicolous copepods), the writer proposes that the term 'commensalism' should be restricted to its literal sense, that of 'sharing the same table'. Thus forms such as *Notodelphys* and *Doropygus* inhabiting the branchial sac should be regarded as true endocommensals, whilst *Lichomolgides*, which lives in the cloacal chamber, could be designated an 'assecla' (Latin *assecla*, a 'hanger-on'). It is obvious that until much fuller information becomes available the dividing line between such categories will remain somewhat arbitrary. However, it seems logical to differentiate, as in the above instance, between copepods living at opposite ends of the host's alimentary canal.

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THE pH OF THE ALIMENTARY TRACT IN NORMAL AND *GIARDIA*-INFECTED CULTURE MICE

By M. H. HAIBA, M.V.Sc., Ph.D. (London)
Faculty of Veterinary Medicine, Giza, Egypt

(With 2 Figures in the Text)

Among the principal intestinal factors that play a part in the host-parasite relationships, the pH values of the parasite's habitat within the host under various conditions are of great significance. As stated previously (Haiba & Williamson, 1952), among the intestinal Protozoa of man and animals, *Giardia* is of much interest owing to its selective localization on the inner epithelial surface or occasionally inside the goblet cells of the upper two-thirds of the small intestine, representing an important area where the main maximal intestinal functions are carried out. Accordingly, the pH of the intestinal wall, as distinct from that of the contents, is likely to be a pertinent environmental factor in giardial infections.

In the literature on this subject, the available information on this point is based only on measurements made in rats. In view of the absence of comparative biological studies on *Giardia* in different hosts, I have therefore undertaken a similar systematic study of the gastro-intestinal pH values in normal and infected mice.

PROCEDURE OF PREPARING MICE AND APPARATUS FOR THE pH DETERMINATIONS

The mice used in these experiments were locally bred animals of both sexes which were 5–6 weeks old. All were kept under the same nutritive and hygienic conditions as the rats used in previous work (Haiba & Williamson, 1952).

Experimental infection with *G. muris* was carried out immediately after weaning. In dealing with delicate animals, with a very narrow oesophageal channel and with infected materials frequently introduced in a relatively thick suspension, it was necessary to select a capillary plastic catheter of suitable size. After a number of trials, a catheter with a bore of 1 mm. was found to be the best, and was used throughout for inoculations. About 2 in. of the tube was smoothed and slightly bent anteriorly, while the other end was attached to the barrel of a 5 c.c. syringe with which the suspension was measured. In manipulating the mice Nelson's technique (1940) was adopted. The capillary catheter was gently introduced into the pharyngeal opening of the mouse, care being taken to avoid injury to the soft membranes or suffocation by introducing some of the material into the lungs. The stream from the syringe was adjusted to the rate at which the mouse laps, in order to prevent spilling, the usual dose of the infected suspension being 0·5 c.c. The mice examined were lightly anaesthetized by subcutaneous injection of Nembutal (1 gr./c.c. in 10% alcohol) in view of its negligible effect on the pH of muscles (Voegtlin, Kahler & Fitch, 1935; Ball, 1939). Several doses of this anaesthetic were tried, and the minimum satisfactory dose adopted for keeping the mouse alive during the experiment was found to be 0·1 c.c. of 1/5 aqueous solution.

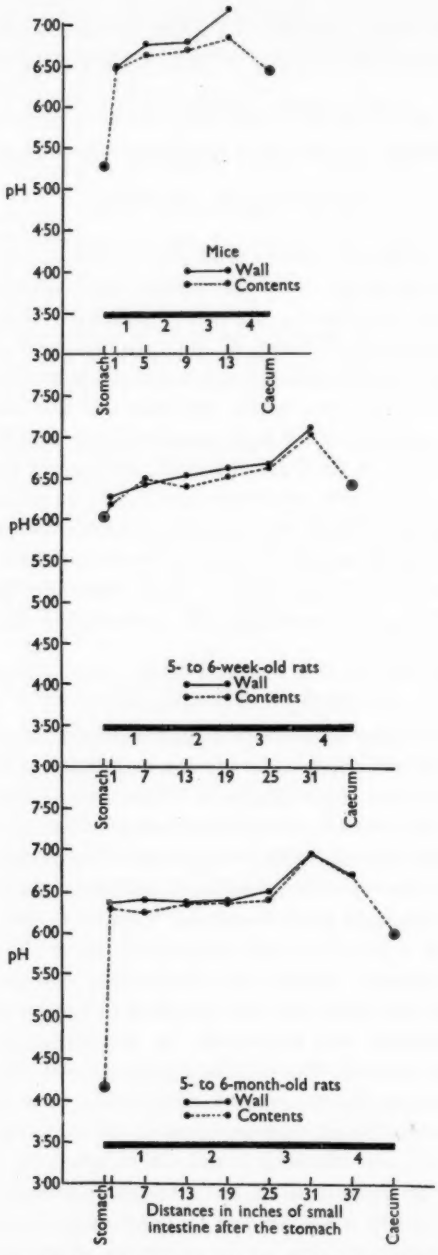

Fig. 1. Average pH determinations, of the contents and wall of the small intestine in normal 5- to 6-month-old albino rats (*Rattus norvegicus albinus*), 5- to 6-week-old albino rats and adult mice (*Mus musculus*).

The determinations in this investigation were made with the same capillary glass electrodes, pH meter, apparatus, connections and the accurate technique which were previously employed for rats (Haiba & Williamson, 1952). The capillary glass electrode of less than 150 μ outer capillary breadth was found to be a convenient and accurate tool for pH determinations on the intestinal wall and contents of mice, for the determinations may be made *in situ* with a minimal disturbance of normal function, thus guarding against erratic results. Readings were made on the contents and then on the wall at 4 in. intervals behind the stomach along the small intestine. The pH values were also estimated for the gastric contents (usually by inserting the electrode into the opaque pyloric glandular portion of the stomach) and for the contents of the large basal portion of the caecum. In the infected group of mice, the presence of parasites was checked by examining the intestine at the end of the pH determinations.

RESULTS

(*a*) *The* pH *of the normal gastro-intestinal contents in relation to that of the wall*

The present determinations revealed that the gastric acidity of mice is intermediate between that of baby and adult rats (Fig. 1). The examinations of normal mice values showed that the pH of the wall was close to that of the contents, exhibiting a conspicuous trend for alkalinity towards the posterior regions of the alimentary tract, especially in the wall, but becoming acid again in the caecum (Table 1 and Figs. 1 & 2). On the whole, the pH values calculated in this investigation of normal mice were found to be approximately within the same range of pH previously obtained for rats (Fig. 1). These results seem to exclude this factor from being the cause of the distribution of *G. muris* throughout the whole length of the small intestine in mice, in contrast to baby rats with nearly the same intestinal length, in which the flagellates are concentrated in the proximal portion of the small intestine. It is possible that other factors, such as the oxygen tension or the bacterial distribution within the two hosts, may be responsible for this difference.

(*b*) *The effect of* Giardia muris *infection on the gastro-intestinal* pH *values in mice*

Differences in the average pH of corresponding areas of the intestinal wall in normal and experimentally infected mice were compared for significance (Table 2). A maximum change in the pH values from normal wall determinations, correlated with *G. muris* infection, in mice was observed approaching significance in the region 5 in. below the stomach (0·05–0·1), and to a lesser degree in the regions at levels of 19 and 13 in. (Table 2 and Fig. 2). This trend towards greater acidity in the more extended infected areas in mice, as compared with the condition found in rats, is correlated with and confirms the foregoing observation regarding the distribution and concentration of *G. muris*, which in mice—owing to unknown factors—occupy the whole length of the small intestine, while having the greatest concentration in the second quarter (Haiba, 1950). As stated in a previous paper (Haiba & Williamson, 1952), the flagellar locomotion of *G. muris* was found to be less

Table 1. *Range and mean* pH *determinations of the contents and wall of the stomach, small intestine (at* 4 *in. intervals) and caecum in normal and* Giardia muris*-infected white mice,* Mus musculus

Condition of mice (all 5–6 weeks old)			Normal	*G. muris*-infected
Stomach	Contents	Range	3·26–6·24	4·98–6·86
		Mean	4·83	5·92
Small intestine				
1 in.	Contents	Range	6·46–6·80	6·28–6·48
		Mean	6·61	6·38
	Wall	Range	6·50–6·74	6·38–6·48
		Mean	6·61	6·43
5 in.	Contents	Range	6·60–6·82	6·36–6·50
		Mean	6·74	6·43
	Wall	Range	6·86–6·94	6·50–6·56
		Mean	6·90	6·53
9 in.	Contents	Range	6·58–6·86	6·32–6·90
		Mean	6·74	6·60
	Wall	Range	6·58–6·98	6·32–6·94
		Mean	6·84	6·63
13 in.	Contents	Range	6·40–7·00	6·84–7·00
		Mean	6·78	6·92
	Wall	Range	6·90–7·52	7·00–7·04
		Mean	7·27	7·02
Caecum	Contents	Range	6·14–6·64	6·22–6·68
		Mean	6·44	6·45

Table 2. *The significance of* Giardia muris*-infections in relation to* pH *values of the walls of small intestine in mice*

Distance (in inches) from the stomach	Mean pH values and significance			
	1 in.	5 in.	9 in.	13 in.
Non-infected mice	6·61	6·90	6·85	7·27
Infected mice	6·43	6·53	6·63	7·02
Differences	0·18	0·37	0·22	0·25
t value at 5% significance	3·182	3·182	3·182	3·182
Observed t value	0·750	2·298	0·471	0·794
Significance level (p)	0·5–0·6	0·05–0·1	0·6–0·7	0·4–0·5

active; this behaviour being the cause of the absence of any significant local inflammatory reaction in the intestine of infected rats. The contrary was observed in the present investigation, since in most of the infected mice examined *post mortem* the small intestine was conspicuously congested concentrically in areas of maximal parasitization. These findings are in agreement with those of Kofoid & Christiansen (1915) and Boeck (1917).

CONCLUSIONS

From Table 1 and Fig. 2 it is seen that in mice *Giardia muris* was found to be concentrated in an area of the small intestine with approximate optimal average wall pH values of 6·38–7·02.

Comparing probabilities of significant levels of pH obtained in this investigation with those previously calculated for rats (Haiba & Williamson, 1952), the author concludes that in mice infected with *G. muris* they are more significant than in rats.

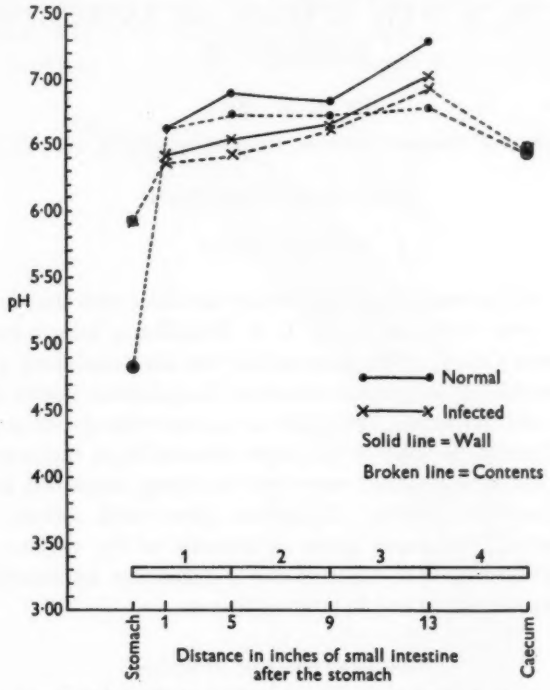

Fig. 2. Average pH determinations at '4' in. intervals of contents and wall of the small intestine in normal and *G. muris*-infected mice.

ACKNOWLEDGEMENTS

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THE BIOLOGY AND HOST RANGE OF *NEOAPLECTANA LEUCANIAE*, A NEW SPECIES OF INSECT-PARASITIC NEMATODE

By J. M. HOY

Entomological Research Station, Palmerston North, New Zealand

(With 5 Figures in the Text)

INTRODUCTION

During October 1945 several pupae, apparently identical with the noctuid *Leucania acontistis* Meyr., were collected by Mr L. J. Dumbleton from a tussock base at Seafield, Ashburton County. One pupa which was dead contained a large number of nematodes resembling in general structure *Neoaplectana glaseri* Steiner (1929). During October and November 1947, 104 larvae and twenty-one pupae of a small tussock moth, *Crambus simplex* Meyr., were collected from the same locality and of these, twenty-six larvae, which were dead or dying, contained nematodes. All pupae were apparently healthy. Nematodes from both sources belong to an undescribed species. This paper gives an account of the various stages of the nematode, its life history and experiments to determine its suitability as a biological controlling factor for soil-inhabiting insects.

DESCRIPTION OF SPECIES

Neoaplectana leucaniae n.sp.

Female (Figs. 1, 2 and 5)

		Nerve ring	Oesophagus	Vulva	Anus	
(6)	—	6·0	8·8	56·3	97·9	1·95 mm.
	—	3·4	4·1	8·5	2·2	

Gravid females (Fig. 1) are stout and almost sausage-shaped, the anterior end tapering more gradually than does the posterior, which terminates in a bluntly conical, short-tipped tail. The pre-adult female has a more slender form, the tail tapering gradually to a point. There is a considerable size variation amongst females, the range observed being from 0·55 mm. to over 4 mm. The 'pygmy' forms, which are disproportionately stout, have also been observed in *N. bibionis* by Bovien (1937), who ascribes them to a possible food deficiency in the host insect.

The cuticle is not striated, nor are bristles present. The vulva is situated near the middle of the body.

The head is neither offset nor recurved, apart from the weak labial tubercules. An *en face* view (Fig. 5) shows three lips, six labial and six cephalic papillae, and amphids.

The buccal cavity is reduced to a small vestibule. The oesophagus (Fig. 2) consists of a cylindrical, moderately thick corpus 17μ across with a slightly swollen posterior portion 26μ across, separated from the terminal bulb by a short isthmus 16μ wide at its narrowest point. The terminal bulb is weak, faintly valvulated, about 36μ across. The nerve ring circles the oesophagus in front of the terminal bulb, and the excretory pore opens to the exterior at a level slightly anterior to the nerve ring.

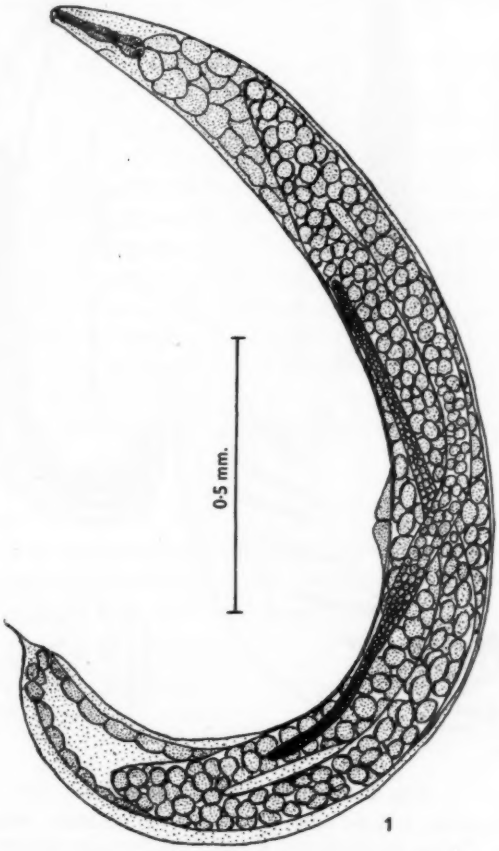
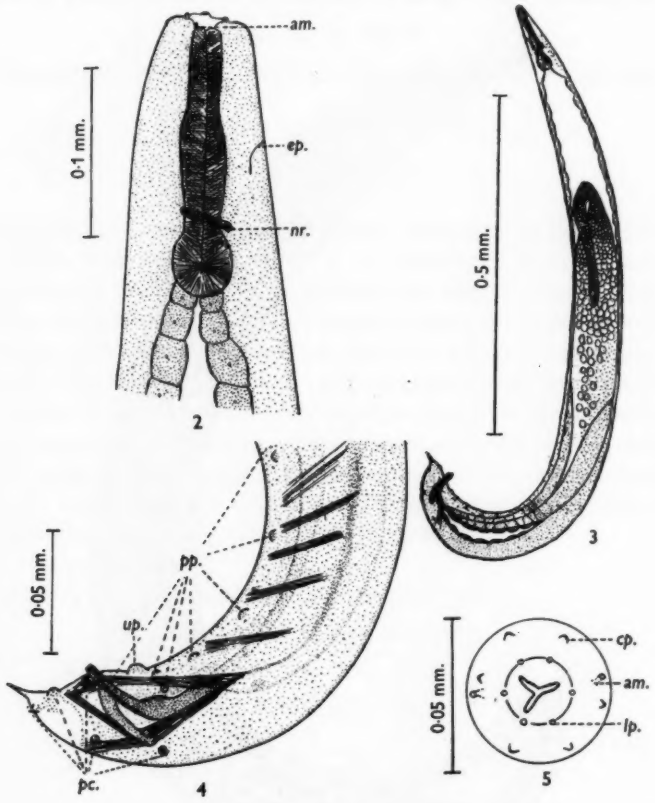


Fig. 1. *Neoplectana leucaniae* n.sp. Gravid female.

The cardia is about 13μ long. In gravid females even the anterior cells of the gut wall behind the cardiac region are pushed to one side by the distended uterus. The cells of the intestinal wall increase in size posteriorly and there are several large anal and preanal cells apparently of a glandular nature. The anal sphincter is well defined at the posterior end of the gut.

The female genitalia are symmetrically placed, the ovaries, which are reflexed, cross one another in the region of the vulva and extend anteriorly to a point half-

way from the vulva to the terminal bulb and posteriorly for a similar distance. The junction of the ovaries and the uterus is not well defined. The uterus extends anteriorly and posteriorly beyond the vagina, sometimes reaching a point anterior to the terminal bulb. The vagina is a short tube opening directly on to the uterus. While in younger females the ovarian tubes are filled with eggs and the uterine



Figs. 2–5. *Neoplectana leucaniae* n.sp.

Fig. 2. Oesophageal region, female. Fig. 3. Male. Fig. 4. Male, cadual region. Fig. 5. Female, *en face* view. Key to lettering: *am.* amphid; *cp.* cephalic papillae; *lp.* labial papillae; *ep.* excretory pore; *nr.* nerve ring; *up.* unpaired caudal papilla; *pp.* paired preanal papillae; *pc.* paired caudal papillae.

tube collapsed, in more advanced specimens the uterus contains a mass of eggs in different stages of cleavage, and when nearly mature the uterus is packed with larvae and segmenting eggs.

Male (Figs. 3, 4)

	Nerve ring	Oesophagus	Mid-point	Anus	
(6)	—	9·7	13·9	—	96·2	0·95 mm.
	—	3·6	4·5	6·9	36·4	

The male (Fig. 3) is smaller and more slender than the female. The tail is bluntly conical, usually ending in a short tip.

Cuticle not striated.

Head similar to that of the female, except that on rare occasions it is offset; amphids present.

Buccal cavity absent; corpus cylindrical, 8μ wide, but proportionately longer than that of female; post corpus slightly expanded to a width of 12μ; oesophagus terminates in a weak, faintly valvulated bulb 18μ across. Excretory pore located closer to head than in the female.

The single testis is reflexed, reaching at times to a distance of 96μ from the terminal bulb. It ends in the vas deferens which forms a well-defined tube, apparently without a seminal vesicle, and joins the narrow ductus ejaculatorius. The paired spicules (Fig. 4) are arcuate ending in an enlarged portion and are not hooked at the tip. The gubernaculum is curved posteriorly, straight anteriorly, ending in a knob which is more strongly offset on the anterior side.

Four sets of muscles can be identified; the retractor spiculi, between the head of the spicules and the subventral muscle field; the protractor spiculi, between the gubernaculum and the base of the spicules; the protractor gubernaculi, between the gubernaculum and the subdorsal muscle field; the levator ani, a short muscle between the cloacal wall and the subventral muscle field.

A number of genital papillae are present: one large unpaired and a series of five to seven preanal subventral papillae and five paired caudal papillae arranged three subdorsal, two subventral.

Larvae

Three larval stages can be distinguished, including a resistant stage termed by Fuchs (1915) the dauerlarva. Measurements were taken for each stage, the following being the averages from four specimens in each case:

		Nerve ring	Oesophagus	Mid-point	Anus	
Newly hatched larva						
(4)	—	15·3	21·6	—	88·6	0·367 mm.
	—	4·4	5·5	5·9	3·4	
Second-stage larva						
(4)	—	13·5	17·8	—	88·9	0·563 mm.
	—	3·9	4·1	5·1	2·9	
Dauerlarva						
(4)	—	12·8	15·8	—	87·2	0·689 mm.
	—	3·9	4·3	4·9	3·4	

Phasmids were observed in all larval stages but were not observed in adults.

The description is based on specimens killed and fixed in Looss's glycerine-alcohol and mounted in either glycerine or glycerine jelly. All slides in the collection of the Entomological Research Station, Palmerston North, N.Z.

Type locality: Seafield, Canterbury, N.Z.

Life history of Neoaplectana leucaniae

The life history of this species closely follows that of other members of the genus, the most outstanding feature being the combination of saprophytic and parasitic habits. In common with a large number of nematodes either parasitic on or associated with insects, *N. leucaniae* has a free-living stage characterized by the retention of the cuticle of the second-larval instar. This retained cuticle forms a loose sheath round the dauerlarva, which is also the invasive stage of the nematode. The occurrence of the dauerlarva is not obligatory, this stage developing only under adverse conditions, particularly where shortage of food occurs within the host insect.

The dauerlarva appears to enter the larval or adult insect orally with food, as it is not equipped to pierce the insect's integument. Once inside the host the nematode undergoes rapid development. The sheath is soon lost and the larva transforms to the preadult stage, in which it is slender, semi-transparent and particularly active. Ecdysis to the adult stage quickly follows.

For egg development the female must be fertilized, this taking place only when the nematodes are mature. The female is ovoviviparous and, as gestation proceeds, a mixture of eggs in different stages of cleavage and first-instar larvae may be seen within the uterus. The normal number of offspring is approximately 250.

The larvae are usually born 24–28 hr. after the female reaches maturity; in the majority of cases the female plays an active role in parturition. The larvae wander within the uterus, occasionally trying to escape through the vulva, the muscles of which suddenly relax to expel a single larva, births occurring singly at intervals ranging from 3 min. to 1 hr. In about 25 % of cases the female dies before all the larvae are born, and in a small number of cases before any larvae are born. The larvae remain in the dead female for periods of up to several days, consuming her body contents and sometimes reaching the second instar before being liberated by their own efforts or the breakdown of the female's integument. The small first-instar larvae, which are slender and almost transparent, grow rapidly, and within 48 hr. transform to the second stage.

The second-instar larvae, which are shorter but stouter than the dauerlarvae, may do one of two things dependent on the environment. When the conditions are favourable for further development the preadult stage follows immediately and the life cycle is completed with three ecdyses, but where the food supply in the host is exhausted or nearly so, the second-instar larva ceases to grow, empties the contents of the alimentary canal and undergoes ecdysis, the old cuticle being retained as a loose sheath. The nematode is now a dauerlarva.

In an insect host which contains an abundant food supply the nematodes continue to develop, completing a generation every 6–7 days where the temperature is 15·5° C., or above. The host insects, particularly larval forms, die in from 2 to 4 days after infection, and once the host is dead the nematodes continue to develop as saprophytes, the carcass of the insect quickly becoming a sac packed with nematodes in all stages of development. The dauerlarvae escape to the exterior following the breakdown of the cuticle of the host, and these free-living forms can

persist for long periods in the natural habitat, the tussock base, while awaiting ingestion by a fresh host.

Culture of Neoaplectana leucaniae *on artificial media*

It was considered worth while to endeavour to rear *N. leucaniae* on similar media to those used for *N. glaseri* by Glaser (1932, 1940*a*, *b*). All attempts to culture the local nematode on dextrose-veal infusion agar with the addition of bovine ovarian substance were unsuccessful. Growth was normal for the first 5–6 days, but bacterial contaminants rapidly suppressed growth from that time on. Similarly, attempts at culture on nutrient agar slopes using sterile rabbit, sheep or beef kidney failed. The same difficulty was experienced by Glaser, McCoy & Girth (1942) with the culture of *N. chresima*, although they found growth satisfactory when the nematodes were sterilized with Milton's solution.

Development of *N. leucaniae* was obtained on a veal pulp medium as used for *N. glaseri* by McCoy & Girth (1938). This medium contains the sodium derivative of methyl-*p*-hydroxybenzoate and formaldehyde as preservatives against fungi and bacteria respectively. Concentrations of 0·05% sodium methyl-*p*-hydroxybenzoate (calculated as methyl ester) and 0·065% formaldehyde held contamination at a low level without impairing growth of the nematode.

The development of *N. leucaniae* on the artificial medium was similar to that in an insect host. The capacity of the nematode to parasitize insects was not impaired after culture on the artificial medium for up to ten generations.

Experimental infection of insects with Neoaplectana leucaniae

The possibility of using *N. leucaniae* as a biological control agent against pest insects in soil was investigated by means of a number of laboratory experiments. Dauerlarvae recovered from dead host insects or from culture plates were suspended in isotonic solutions and added to partially sterilized soil. No attempt was made to standardize nematode dosage since the experiments were designed solely to show whether or not infection could occur and the percentage infection obtained is not necessarily related to that which could be expected in the field. Infection was demonstrated by dissection and recovery of nematodes identifiable as *N. leucaniae*.

The first experiment was designed to demonstrate the number of melolonthid species which could be infected by *N. leucaniae*. Third-instar larvae of all the available melolonthid species were tested, identifications of species being based on descriptions of Hoy & Given (1952). The species used were *Costelytra zealandica* (White), *Odontria communis* Given, *O. nitidula* Broun, *O. autumnalis* Given, *O. striata* White, *Pyronota festiva* (Fab.), *P. inconstans* Brookes, and *P. inconstans* var. A. Given. Table 1 shows the results obtained.

Similarly, when adult beetles of these species were confined in soil containing dauerlarvae of *N. leucaniae*, all species were infected. The time taken to kill adult melolonthids is longer than that required to kill larvae, ranging from 9 to 14 days; a similar time difference occurs when using *N. glaseri* against the same species (Dumbleton, 1945).

In addition to these species, *N. leucaniae* will parasitize larvae of *Oxycanus*

Table 1. *Infection of larval* Melolonthinae *by* Neoaplectana leucaniae

Host larvae	No. used	No. infected	Time in days to first death at 75° F.
C. zealandica	6	6	2
O. communis	3	2	4
O. nitidula	10	9	3
O. autumnalis	4	3	3
O. striata	5	5	3
O. smithii	6	6	4
P. festiva	1	1	2
P. inconstans	2	2	3
P. inconstans var. A.	1	1	4

cervinata Walk., *Persectania ewingi* Westw., elaterids, asilids, *Pericoptus* species and an unidentified soil inhabiting weevil. Earthworms confined in soil containing *N. leucaniae* were recovered alive and apparently healthy at the end of 90 days. The worms were not dissected.

The possibility of natural spread of the nematode by means of infected beetles, in the event of the utilization of *N. leucaniae* as an agent for the biological control of *C. zealandica*, was investigated on a laboratory scale. One hundred beetles of mixed sexes of *C. zealandica* were confined in sterilized soil infested with dauerlarvae of *N. leucaniae*. The soil was watered from time to time to maintain the moisture level, and rose leaves were added daily as food for the beetles. After 4 days a trap was inserted to collect beetles flying from the soil. Thirty individuals were thus trapped and removed to sterilized soil in a second container. Both containers were held at summer room temperatures. As the beetles died in each container they were dissected for nematodes. Out of seventy beetles dying in or on the soil in the original container, twenty-two were infected with *N. leucaniae*. Of the thirty beetles which flew from infected soil, three contained nematodes and all were dead at the end of 17 days, which was the duration of the experiment.

This indicates that adults of *C. zealandica* can become infected from soil carrying the dauerlarvae of *N. leucaniae* and that a small percentage of the flying beetles can carry the infection to fresh foci.

Ability of Neoplectana leucaniae *dauerlarvae to survive in soil*

The above results indicated that, provided *N. leucaniae* dauerlarvae are capable of surviving in the soil under adverse conditions such as fluctuating soil temperature, soil moisture and low host populations, this nematode might be a useful adjunct to the attempted control of *C. zealandica* by *N. glaseri*. In pot experiments under these conditions no dauerlarvae of *N. leucaniae* could be recovered from the soil after 3 months. Under similar conditions dauerlarvae of *N. glaseri* have survived for over 4½ years. In its natural habitat—the tussock base—*N. leucaniae* appears to survive for long periods in the absence of host insects. Since this lack of persistence in soil seriously limited the use of *N. leucaniae* in biological control work, the artificial culturing for the control of *C. zealandica* was discontinued.

SUMMARY

The morphology and life history of a new species of insect-parasitic nematode, *Neoplectana leucaniae* are described. This nematode, which may be cultured on a veal pulp medium, was used in a number of experiments against larvae and adults of New Zealand *Melolonthinae*, and was found capable of parasitizing all test hosts used. Lack of persistence of the nematode in soil makes it unsuitable for use in the biological control of soil-inhabiting pest insects.

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A COMPARATIVE STUDY OF DIFFERENT TYPES OF VIRUSES AND THEIR CAPSULES IN THE POLYHEDROSES AND GRANULOSES OF INSECTS

By KENNETH M. SMITH and N. XEROS

Plant Virus Research Unit (Agricultural Research Council), Molteno Institute, University of Cambridge

(With Plates XIII and XIV)

It has recently been shown (Smith & Wyckoff, 1950) that, in addition to the classical type of nuclear polyhedral disease of lepidopterous larvae, there exists a second type of polyhedrosis with spherical virus particles instead of rods. This was demonstrated in the case of two tiger-moth larvae, *Arctia villica* and *A. caja*. The polyhedra of this type of disease do not dissolve fully when treated with weak alkali but leave behind a honeycomb-like shell pitted with round holes. We have also shown (Xeros, 1952, Smith & Xeros, 1953*a*) that in all our material these polyhedra occur in the cytoplasm and not in the nuclei and are confined to the cells of the midgut.

In our examination in the electron microscope of polyhedra from different cytoplasmic polyhedroses treated with different strengths of sodium carbonate, we have experienced great difficulty in seeing the viruses themselves. This is quite contrary to our experience with the large numbers of nuclear polyhedroses which we have examined by similar methods. In these it is usually fairly easy to observe the virus rods liberated from the polyhedra by the alkali.

We therefore came to the conclusion that this technique was inadequate for these cytoplasmic polyhedra, and that in most cases the polyhedral protein was comparatively resistant to the action of alkali and the virus particles were dissolved away. This might account for the numerous empty holes in the polyhedral shell. Other interpretations however are also possible. For example, it is arguable that the virus spheres are embedded within a skeletal framework of polyhedral protein very resistant to alkali dissolution and around which more soluble protein is found, or that the virus spheres have been overlooked because of an unexpectedly small size.

We therefore turned our attention to the examination of thin sections of fixed larvae infected with cytoplasmic polyhedroses. We have examined a number of different species with this type of disease, including *Bombyx mori*, *Abraxas grossulariata*, and *Phlogophora meticulosa*. It is with the larvae of *P. meticulosa* with which we are concerned here. Thin sections of the gut revealed an interesting state of affairs. If the sections through the polyhedral crystals were thin enough, they could be seen to consist of a mass of spherical bodies approximately 60 mμ in diameter, held together in a matrix of polyhedral protein (Pl. XIII, fig. 1). Under higher magnification each of these spherical bodies could be seen to be composite, consisting of a number, usually four, of very small units each about 15 mμ in

diameter (Pl. XIII, fig. 2). The composite bodies were also to be found loose in the cytoplasm, together with a certain number of the small single units (Pl. XIII, figs. 1, 2).

The exact significance of these phenomena in *P. meticulosa* is not at present completely clear. However, the size of the spherical bodies from *P. meticulosa* (*ca.* 60 mμ) is about the same as for those from *Arctia villica* (*ca.* 65 mμ) (Smith & Wyckoff, 1950; Bergold, 1952), and which have been regarded as the virus by these authors. As we have seen, the spherical bodies of *Phlogophora meticulosa* are not simple spheres but compound, and the pictures of those from *Arctia villica* suggest that in this species too they may be compound. The groups of small spheres apparently held together in a capsule material in *Phlogophora meticulosa* might be compared to the bundles of three or four or more virus rods, also in a capsule, which occur in the nuclear polyhedroses of *Lymantria monacha* (Pl. XIV, fig. 11) and of *Abraxas grossulariata* (Smith & Wyckoff, 1951), etc. The virus rod or rods enclosed in capsules are embedded in further protein which makes up the polyhedra. In the nuclear polyhedrosis of *Bombyx mori* the overwhelming majority of the rods are found singly, yet even so each virus rod is found enclosed within a capsule further embedded in the polyhedral protein (Smith & Xeros, 1954; also Pl. XIV, fig. 9). The mass of polyhedral protein may therefore be regarded as an outer capsule within which are embedded virus particles enclosed in inner capsules. This phenomenon of the virus being surrounded with a capsule and again further enclosed within an outer deposit of protein is very characteristic of the polyhedroses and is well illustrated by the electron micrographs of the polyhedroses of *B. mori* and *Lymantria monacha* (Pl. XIV, figs. 9, 11). In our electron microscope examination of the granulosis disease of the nettle grub *Natada nararia*, a caterpillar which feeds on the tea plant in Ceylon, we have found what appears to be a similar phenomenon. The virus surrounded by its intimate membrane, which is present also around the rods of the nuclear polyhedroses, appears to be enclosed within an inner capsule and further surrounded by an outer capsule. In this particular granulosis each inner capsule encloses only one rod, as in the case of the polyhedrosis of *Bombyx mori*. However, whilst in the polyhedrosis of *B. mori* the outer capsule or polyhedral matrix encloses numerous virus rods each within their inner capsule, the outer capsule in the granulosis of *Natada nararia* encloses only one rod with its inner capsule.

Suspensions of granules from granulosis-infected *N. nararia* were mounted on grids and, after drying, treated with sodium carbonate solution of varying strengths and for varying times. The micrographs (Pl. XIV, figs. 5–8) show the course of dissolution of the granules (*ca.* 360 × 200 mμ in size). First the outer capsule disintegrates and partially dissolves, leaving an expanded residue on the grid. Within this outer capsule a second inner capsule (*ca.* 310 × 80 mμ) is revealed. As the action of the alkali proceeds this inner capsule in turn dissolves to reveal the much thinner virus rod (*ca.* 290 × 45 mμ). Furthermore, as the virus rod is subjected to alkali disintegration and dissolution, the intimate membrane enclosing the virus material itself is seen. In some rods the virus material is dissolved away from each end, and only the middle part of the virus material remains. In others, the virus

material is dissolved out of the middle region of the rod, leaving two small remnants, one at each end. The only membrane, as distinct from capsules, of whose existence we are certain in this case is the intimate membrane surrounding the actual virus material. No membrane surrounding the inner or outer capsules was observed.

The formation of the granules of *N. nararia* takes place in the nuclei of the hypodermis and fat body. The appearance of the nuclei of diseased cells is similar to that of diseased cells of *Junonia coenia* (Steinhaus & Thompson, 1949) and of *Sabulodes caberata* (Hughes & Thompson, 1951). The cytoplasm and nuclei of diseased cells grow enormously and the chromatic material of the cytoplasm and especially of the nucleus greatly increases. There is no formation of a central chromatic mass and empty ring zone as occurs in the nuclear polyhedroses. Within the mass of nuclear material, round to sausage-shaped areas of more lightly staining chromatic material develop and gradually lose all their capacity for taking basic stains, finally staining a weak pink with Giemsa. Between these bodies cords of deep staining chromatic material remain, which are disrupted and lost as the enormously enlarged nuclei disintegrate. The sausage-shaped bodies stain weakly with bromophenol blue if not pre-treated with HCl, but stain intensely after such pre-treatment. The protein of the chromatic material stains moderately with bromophenol blue and somewhat more intensely after HCl pre-treatment. At about the time the nucleus disintegrates many of the sausage-shaped bodies can be seen to be breaking up into minute granules similar in all respects to the granules isolated from diseased caterpillars. No sections of *Natada nararia* have yet been examined with the electron microscope. However, there is no doubt that the sausage-shaped bodies are aggregates of the granules. Pl. XIV, fig. 3, a photograph on the optical microscope of a section of an infected larva of *N. nararia* in a moribund condition, shows nuclei in different stages of enlargement.

The granulosis virus diseases were first described by Paillot (1926) and later by Steinhaus (1947) and Bergold (1948). In these diseases the inclusion bodies are far smaller than the polyhedra of the polyhedroses. It was suggested by Steinhaus that each granule might contain a single virus particle. Bergold (1948) and Steinhaus, Hughes & Wasser (1949) published the first electron micrographs of granules treated with weak alkali. In these first descriptions the pictures were interpreted to mean that one virus particle was released from each capsule, leaving a rod-shaped cavity in the granule substance. A closer examination of the pictures, however, suggests that a different interpretation is equally, if not more plausible, namely that alkali treatment caused the dissolution of an outer capsule, leaving two virus rods each within an inner capsule, which together with the outer capsule had constituted a single granule. Fig. 2D of the paper of Steinhaus *et al.* (1949) and fig. 164B in Steinhaus's book (1949) definitely suggest that there are two rods per capsule in the granulosis of *Peridroma margaritosa*. The intermediate stages of dissolution are, however, lacking, and it is not clear whether each rod is in a separate inner capsule or whether both rods are within one inner capsule, if there are indeed two rods in each capsule. Bergold (1952, 1953) states that only occasionally is there a 'double rod', i.e. two rods, in one granule; but the over-

whelming majority of his published pictures of *Cacoecia murinana* granules, including those published in 1948 when he supposed there was only one virus rod per granule, show two virus rods per granule. The possibility that the granules of *C. murinana* may have two virus rods per granule has also been pointed out by Tokuyasu (1953). Pictures of all stages of dissolution of the granules of *C. murinana* and of *Peridroma margaritosa* are required. The pictures would probably be easier to interpret if they were of granules deposited on grids and dissolved *in situ* to all stages up to the visualization of the intimate membranes.

Steinhaus & Thompson (1949) described a new granulosis of *Junonia coenia* in which there was without doubt only one virus rod per granule. They showed pictures of the virus still enclosed in 'some of the granular material' but did not identify this material as an inner capsule. Thompson (1951), in reporting a granulosis of *Pieris rapae*, gave no details of the inner morphology of the granules. Wasser & Steinhaus (1951) reported a granulosis of *Argyrotaenia velutinana* stating that apparently one rod was enclosed within each capsule. In their illustration of partially dissolved granules (fig. E) however, the distribution of the electron adsorption suggests that two rods may be present in each capsule. Hughes & Thompson (1951) and Hughes (1952) showed there was one virus rod per granule in the granulosis of *Sabulodes caberata*. Tanada (1953) described with electron micrographs a granulosis of *Pieris rapae*. This disease has one virus rod in each granule. Tanada states that there was a great variation in the width of the virus rods (40–100 mμ). It is clear from the published pictures, however, that this huge variation is not entirely due to variations in the width of the virus rods themselves, but that the virus particles are about 40 mμ wide, and those bodies taken as very wide rods by Tanada are virus rods still surrounded by their inner capsules, and having a diameter of about 90 mμ. The intact undissolved capsules are about 185 mμ in diameter, and the flattened intimate membranes about 50 mμ in diameter, that is, wider than the virus rods. It is important that the virus rods should be freed of capsule material before measurement of their diameter. Whether they are free or not free of inner capsule material can be checked by observing the diameter of the collapsed intimate membranes of completely dissolved rods.

From our own and other published evidence, we conclude that the virus rods of both the nuclear polyhedroses and the granuloses become enclosed in an inner capsule and an outer capsule which in the case of the polyhedroses is the polyhedral crystal. We have presented evidence that a similar condition may hold for the cytoplasmic polyhedroses. Moreover, the inner capsule in these various inclusion-body diseases may include one or more virus particles. The outer capsule may enclose many virus bundles as in the polyhedroses or one or two rods with their inner capsules as in the granuloses. Aberrant long granules have been featured by Bergold (1948), Steinhaus *et al.* (1949) and Tanada (1953). Whether these forms are derived from nuclei which had not completed their normal cycle of changes to disintegration is not known. Neither is it known whether they contain several normal-sized or one very long virus rod.

The evidence from the polyhedroses is the strongest evidence for the existence of an inner capsule distinct from the outer polyhedral protein. In what way the

proteins of the outer and inner capsules are related, and what are their similarities or differences, is however not known.

We have shown (Smith & Xeros, 1953*b*, 1954) that the inner capsule is not a 'developmental membrane', but a capsule acquired by the virus rod after its formation and after it has been freed into the ring zone. This we demonstrated in sections of polyhedrosis-diseased *Bombyx mori* and later in *T. bisselliella* (unpublished). The empty intimate membranes should not be confused with the inner capsules or any membrane which might bound the inner capsules, a confusion which seems to exist in several papers. The evidence of Hughes (1952) shows that in the granulosis of *Sabulodes caberata*, the capsule is acquired after the formation of the virus rod. However, we have been unable from his data to decide whether outer and inner capsules are acquired together or whether the inner capsules form first and the outer capsules later. In the polyhedrosis of *Bombyx mori* we have no evidence that the deposition of the inner capsule material round the virus rod occurs in a polar fashion, though rods half capped with inner capsule have been observed by us both in this species and *Lymantria monacha*, but only on virus rods dissolved out of polyhedra and their precise significance is not clear. In sections of *Bombyx mori* material fixed with osmic acid, however, we have observed virus rods within an inner capsule and surrounded by diffuse uncrystallized polyhedral protein (Pl. XIV, fig. 10).

We have also shown (Smith & Xeros, 1954; see also Pl. XIV, fig. 11) that the so-called spherical developmental forms of the virus described by Bergold (1950, 1952, 1953), are not spherical forms at all but bundles of approximately half length rods enclosed within an inner capsule, e.g. in *Lymantria monacha*, *L. dispar*, *Bombyx mori*, etc. Such forms also exist in the granuloses, e.g. of *Natada nararia*, *Cacoecia murinana* (plate 4, Bergold, 1950), *Argyrotaenia velutinana* (fig. E, Wasser & Steinhaus, 1951), etc. The proportion of half-length to normal-sized rods varies from one species to another, and among those which we have examined it is highest in *Lymantria monacha*, next in *L. dispar*, with a very few half-sized rods in *Bombyx mori*, *Abraxas grossulariata*, etc. We have also described in sections of *Bombyx mori* infected with its nuclear polyhedrosis extremely long virus rods of a size we have never observed among rods obtained from polyhedra. Similar long rods which appeared to be breaking up into two more normal-sized rods in the ring zone, have also been observed. We have, therefore, put forward the idea that the virus rods tend to break and that the two most stable sizes are the full and half lengths. Finally, the V-shaped forms described by Bergold (1950) and Bird (1952) are clearly in almost all cases either two half-length rods within one inner capsule, or two bundles of half-length rods, each bundle within its own inner capsule and the two bundles lying alongside each other at an angle. These forms should not be confused with slightly curved rods which are sometimes obtained in the various preparations.

We therefore consider that almost the entire evidence brought forward by Bergold (1950) to illustrate a life cycle in these viruses has been invalidated. Unusual forms found in preparations should be examined on their own merits and not referred to uncritically as further evidence illustrating the supposed life cycle.

We urge the abandonment of the term 'developmental membrane' and the adoption of the term 'inner capsule' for the capsule of material immediately surrounding the rod and its intimate membrane.

We are indebted to Mr J. Lamb and Dr C. A. Loos of the Tea Research Institute, Ceylon, who kindly supplied us with a number of larvae of *Natada nararia* infected with a granulosis virus. Miss S. Vernon-Smith took the electron micrographs. Mr S. Frey took the photographs on the optical microscope.

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EXPLANATION OF PLATES XIII AND XIV

PLATE XIII

Fig. 1. Section of larva of the angleshades moth, *Phlogophora meticulosa*, infected with a cytoplasmic polyhedrosis. Note the two polyhedra which are very thinly cut and which consist of a mass of spherical virus bodies about 60 mμ in diameter in a loose matrix of polyhedral protein. Free virus particles can be seen in the centre. × 27,500.

Fig. 2. The cytoplasmic virus bodies shown at a high magnification. Note that the bodies are composite, consisting of 4 or more units, each of which measures about 15 mμ in diameter. × 50,000.

PLATE XIV

Fig. 3. Section through a moribund larva of *Natada nararia*, infected with granulosis; note the greatly enlarged nuclei of the fat body. Giemsa stain, × 650.

Figs. 4*a*, *b*. Unstained and untreated smear of recent preparation of *Lymantria dispar* polyhedra, showing large numbers of virus bundles. In some polyhedra the virus bundles are apparently absent. Oil-immersion lens, same field at two different levels.

Fig. 5. Untreated granules of *Natada nararia*, infected with a granulosis disease. × 25,000.

Figs. 6, 7. Similar granules after sodium carbonate treatment, showing inner and outer capsules, and one free virus rod. × 25,000.

Fig. 8. Two virus rods from the same granules showing the intimate membranes where partial dissolution of the virus material has occurred. × 25,000.

Fig. 9. Virus rods of *Bombyx mori* dissolved out of a polyhedral body with sodium carbonate: note that three virus rods have lost their inner capsules. × 25,000.

Fig. 10. *B. mori*, section through the nuclear ring zone at a late stage of infection; note polyhedral body in upper right-hand corner, and capsules in process of forming round the virus rods. × 24,000.

Fig. 11. Virus contents of polyhedral body from *Lymantria monacha* showing bundles of normal and half-sized rods, with and without their inner capsules. × 25,000.

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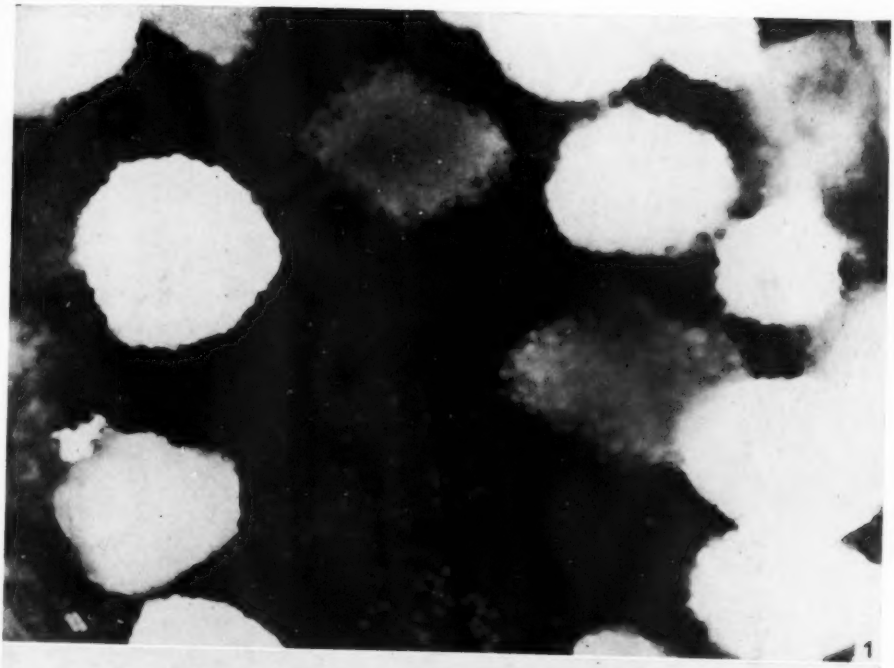

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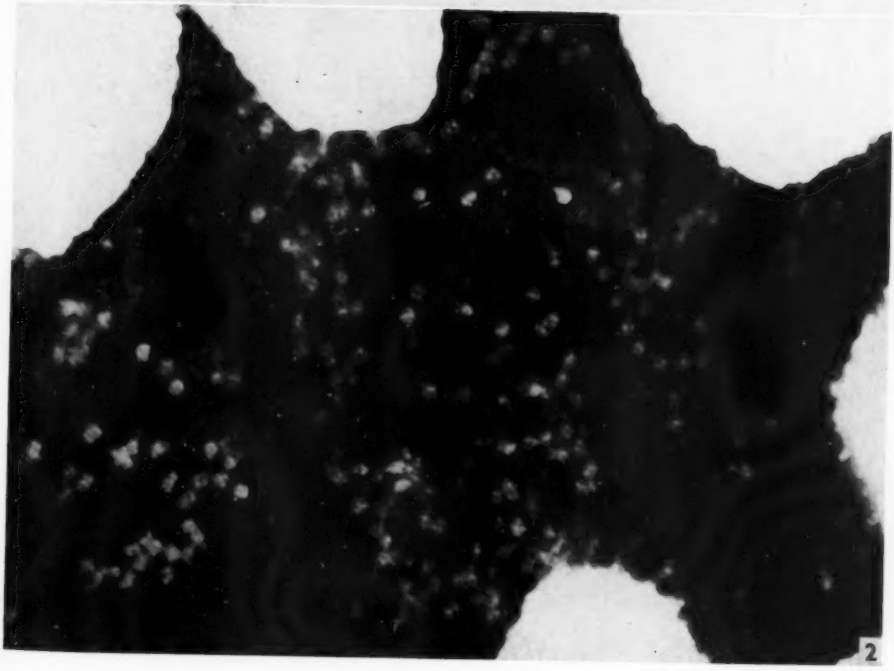

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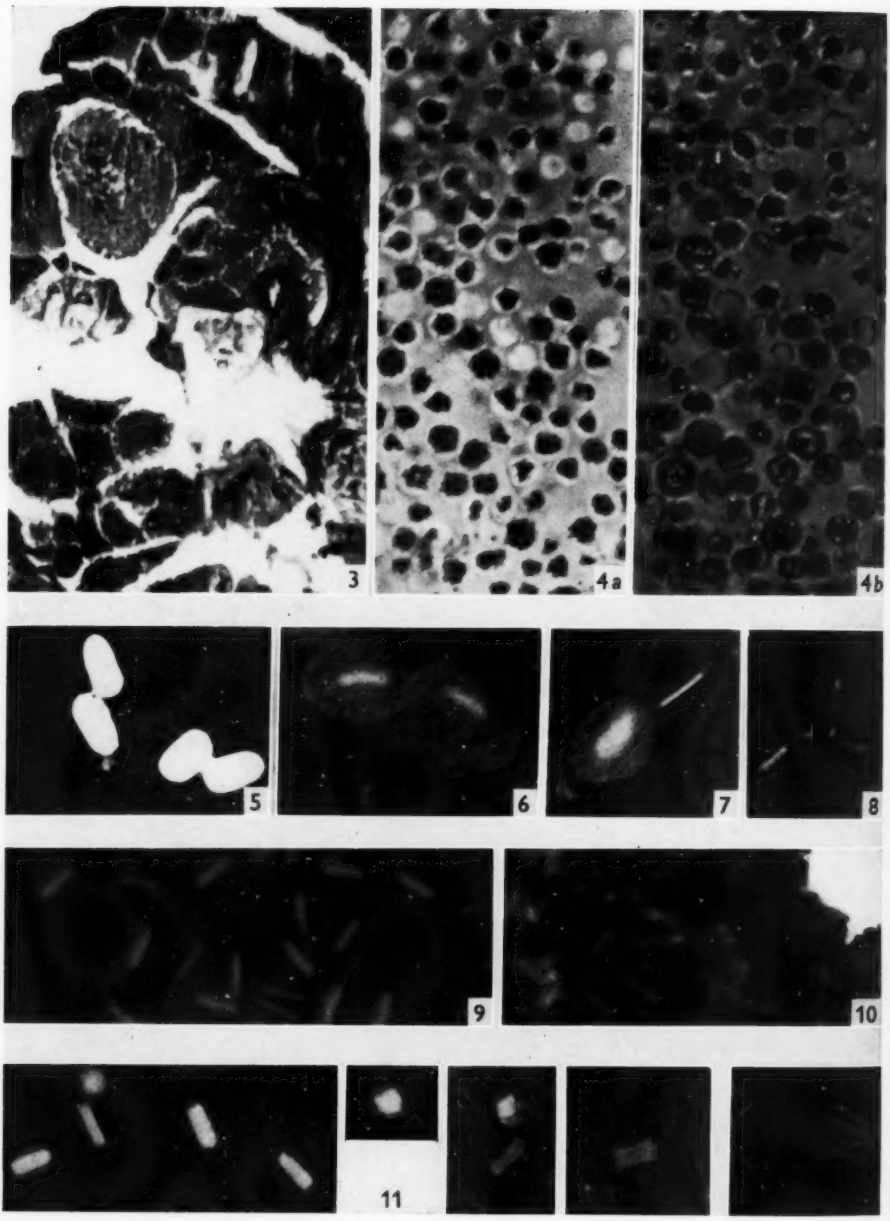
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THE COMPLEMENT-FIXATION TEST IN BILHARZIASIS

I. THE VALUE OF DIFFERENT EXTRACTS OF *SCHISTOSOMA MANSONI* AND *FASCIOLA HEPATICA* WORMS AS ANTIGENS

By M. ELIAKIM and A. MICHAEL DAVIES*

The Medical Corps, Israel Defence Forces, M.P.O. 152 and the Israeli Institute for Biological Research, Ness Ziona, Israel

(With 2 Figures in the Text)

Extracts of adult *Schistosoma mansoni* worms in Coca's solution form reliable antigens in the serodiagnosis of bilharziasis (Davies, Eliakim & Gilon, 1953); aqueous extracts (Hayami & Tanaka, 1910; Bozicevich & Hoyem, 1947) and alcoholic extracts (Yoshimoto, 1910) show complement-fixing properties. Alcoholic extracts of *Fasciola hepatica* worms possess little activity in this respect (Bettencourt & Borges, 1922; Le Bas, 1923–4) and behave more as Wasserman than as bilharzial antigens. Khalil (1925), on the other hand, claimed good results with both alcoholic and aqueous extracts of these worms, but was obliged to exclude sera of luetic patients from his series.

The Expert Committee on Bilharziasis of the World Health Organization (1953) has stressed the importance of study of the nature of the antigen in serological tests for bilharziasis with a view to the development of a more specific and/or sensitive test. The present study was undertaken to compare the efficiency of different extracts of *S. mansoni* and *F. hepatica* worms as antigens in the complement-fixation test (C.F.T.) for bilharziasis.

MATERIAL AND METHODS

A. *Sera*

(1) *Positive series*

Sera were obtained from forty-five male and three female hospitalized patients with proven chronic bilharziasis. In six patients the disease was of 1 year's duration, in the others of 3 years' at least. Thirty-seven patients were infected with *S. mansoni*, nine with *S. haematobium* and two with both. Ages varied from 15 to 29 years, the majority being between 19 and 23.

Twenty-four patients had received no treatment up to the time of bleeding. Of the other twenty-four, eleven were examined during the course of treatment (having received at least 1 g. of sodium antimonyl tartrate), a further seven, also examined during treatment, had received previous, unsuccessful therapy, and the remaining six, who had been treated 10 months previously, were clinically cured.

(2) *Control series*

This consisted of the sera of thirty-one male and twenty-nine female healthy subjects, who had recently immigrated from European countries free of bilharziasis, and were of similar ages to the positive series.

The sera were preserved by the addition of 1:10,000 sodium merthiolate and had been stored at $-20°$ C. for 4–9 months.

* Now at Department of Hygiene, Medical School, Hebrew University of Jerusalem, Israel.

B. *Worms*

(1) *Schistosoma mansoni*

Worms were removed from mice infected with an Egyptian strain (Standen). They were immersed in normal saline on the bench during the period of autopsy (2–180 min.), washed twice in sterile warm saline, rinsed briefly in distilled water and dried in the vacuum desiccator in the refrigerator. When dry, the worms were ground in a mortar, kept in the cold and then sealed as a dry powder *in vacuo*.

(2) *Fasciola hepatica*

Worms which had been preserved as a dry powder for 18 months were obtained from O. D. Standen through the courtesy of Professor G. Witenberg.

C. *Extracts*

Except where otherwise stated, the volumes of all extracts were adjusted to give a 1:100 dilution of the original worm powder, and were stored in the refrigerator at 4° C. All extractions for comparative tests were performed at the same time and tested within 1–2 days of preparation.

I. *Coca's solution*

Thirty mg. of worm powder were suspended in 3 ml. of Coca's solution (0·5 g. NaCl, 0·05 g. $NaHCO_3$, 0·4 g. phenol, distilled water to 100 ml.), giving a dilution of 1:100. Extraction was performed at 37° C. for 1 hr. with vigorous shaking at 10 min. intervals, and the suspension allowed to sediment overnight at 28° C. It was then centrifuged at 2000 r.p.m. for 20 min., decanted and the supernatant adjusted to 3 ml. with Coca's solution. The final pH was 7·35.

II. *Alcohol-ether*

Thirty mg. of worms were suspended in 3 ml. of a mixture of equal parts of absolute alcohol and ethyl ether, and were extracted in a sealed tube by the above method, the plug being exchanged for cotton-wool during the period at 28° C. 0·6 ml. of extract remained after centrifugation and this was adjusted to 3·0 ml. with 0·85% saline. The sediment was very rapidly washed twice with saline and the washings added to the extract giving a final concentration of 1:300.

III. *Coca's solution extract of alcohol-ether insoluble residue*

The residue of extract II was further extracted in Coca's solution as described above.

IV. *Acetone*

Thirty mg. of dried worm powder were extracted for 30 min. at 37° C. by three lots of 10 ml. pure acetone, vigorously shaking every 10 min. The supernatant portions after centrifugation were pooled, dried in the vacuum desiccator at room temperature, and the residue resuspended in 3 ml. merthiolated saline.

V. *Alcohol extract of acetone insoluble residue*

The acetone insoluble centrifugate of extract IV was further extracted in 3 ml. absolute alcohol as described for extract I. The alcohol was then removed by

evaporation in the vacuum desiccator and the residue resuspended in merthiolated saline to a dilution of 1:100.

VI. *Formamide extract*

One hundred mg. of worms were extracted in formamide at 150° C. (Fuller, 1938), the polysaccharide extract then precipitated with an excess of acetone and resuspended in 10 ml. saline.

VII. *Acetone precipitate of alcohol-ether soluble fraction*

Three ml. of extract II were concentrated to 2 ml. in the vacuum desiccator and 2 ml. pure acetone added. The mixture was shaken vigorously for a few minutes and then centrifuged for 30 min. at 3000 r.p.m. The minute precipitate so obtained was resuspended in merthiolated saline to give a 1:100 suspension with regard to the original worm powder.

D. *Complement-fixation test*

The C.F. test previously described (Davies & Eliakim, 1954) was used throughout. Antigen and complement titrations were performed on the day of the test and the titre of antigen was taken as the highest dilution to give full inhibition of haemolysis with pooled positive serum and 2 M.H.D. of complement. Results were graded as: ± (< 25 % inhibition of haemolysis), + (25–50 %), + + (50–75 %), + + + (75–100 %), and + + + + (100 %). All comparative tests were performed simultaneously.

E. *Immunization of rabbits*

Three albino rabbits, weighing 2·4–2·6 kg., were immunized individually with extracts I, II and VI, a fourth rabbit serving as control. The method adopted was to give four weekly intraperitonal injections of 0·5 ml. of 1:100 antigen dilution mixed with an equal volume of 2 % sterile sodium alginate. Leaving the needle *in situ*, 0·2 ml. of sterile 2 % calcium chloride were injected from a second syringe yielding an antigen depot in the gel so formed. One month later a fifth injection was given. The rabbits were bled from an ear vein at the commencement of the experiment, and 10 days after the last injection, and the sera preserved in the usual way. Three months later, a sixth injection was given, followed after 10 days by skin tests, each rabbit being tested on its shaven abdomen with each of the three antigens at a dilution of 1:1000. The technique of the skin test followed that previously described (Davies & Eliakim, 1954).

RESULTS

A. Schistosoma mansoni *extracts*

(1) The comparative efficiency of extracts I–VI, when titrated against the same pooled positive serum, is shown in Table 1. Extracts I, II and V are seen to possess strong complement-fixing activity, the last two being more sensitive.

Extract III had little antigenic potency while extracts IV and VI had none in a dilution of 1:250.

(2) The four extracts showing activity (I, II, III and V) were tested with the sera of twenty-four untreated and twenty-four treated patients (Fig. 1). Extract I is

Table 1. *Titration of different extracts of* Schistosoma mansoni *worms with pooled serum of patients with bilharziasis*

Extracts ...	I			II			III		
	Complement (M.H.D.)			Complement (M.H.D.)			Complement (M.H.D.)		
Antigen dilution	2	3	5	2	3	5	2	3	5
1:250	++++	++++	++++	++++	++++	++++	++++	++++	+++
1:500	++++	++++	++++	++++	++++	++++	++++	+++	+
1:1000	++++	+++	±	++++	++++	++	+	±	–
1:1500	++	±	–	++++	++++	+	–	–	–
1:2000	++	–	–	++++	+	±	–	–	–
1:2500	+	–	–	++	+	–	–	–	–
1:3000	–	–	–	+	±	–	–	–	–
1:3500	–	–	–	+	–	–	–	–	–
1:4000	–	–	–	–	–	–	–	–	–
1:4500	–	–	–	–	–	–	–	–	–
1:5000	–	–	–	–	–	–	–	–	–

Extracts ...	IV			V			VI		
	Complement (M.H.D.)			Complement (M.H.D.)			Complement (M.H.D.)		
Antigen dilution	2	3	5	2	3	5	2	3	5
1:250	±?	±?	–	++++	++++	++++	–	–	–
1:500	±?	–	–	++++	++++	++++	–	–	–
1:1000	–	–	–	++++	++++	++++	–	–	–
1:1500	–	–	–	++++	++++	+	–	–	–
1:2000	–	–	–	++++	+	–	–	–	–
1:2500	–	–	–	++	+	–	–	–	–
1:3000	–	–	–	+	+	–	–	–	–
1:3500	–	–	–	+	+	–	–	–	–
1:4000	–	–	–	+	–	–	–	–	–
1:4500	–	–	–	±	–	–	–	–	–
1:5000	–	–	–	–	–	–	–	–	–

Key. Extracts: I, Coca's solution; II, alcohol-ether; III, Coca's solution of alcohol-ether insoluble residue; IV, acetone; V, alcohol of acetone insoluble residue; VI, formamide.

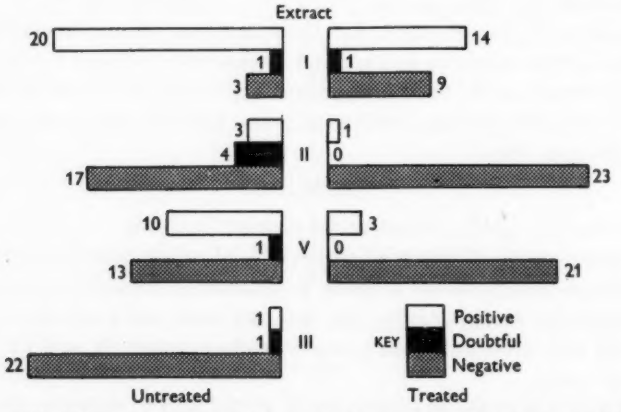

Fig. 1. C.F.T. with different extracts of *S. mansoni* worm tested against sera of twenty-four treated and twenty-four untreated cases of bilharziasis.

evidently the most specific, diagnosing twenty in the non-treated and fourteen in the treated series. For extract II, the numbers were three and one respectively and for extract V, ten and three. Extract III gave a positive result in only one of twenty-four sera of untreated patients and in none of seven (not shown in the chart) sera from the treated series.

Of the nine sera from patients with *S. haematobium* infestation six were in the treated series. Extract I gave correct results with three of the treated and two of the non-treated patients, extract II gave negative results with all sera, and only one serum of a treated patient which, though negative with the other extracts, was positive with extract V. Three sera which were tested against extract III reacted negatively.

None of the fifty control sera gave a positive result with any of the four antigens.

Table 2. *Titration of acetone precipitate of alcohol-ether soluble fraction with pooled serum of patients with bilharziasis*

Antigen dilution	Complement (M.H.D.) 2	3	5
1:1000	+ + + +	+ + + +	+ + + +
1:1500	+ + + +	+ + + +	+ + +
1:2000	+ + + +	+ + + +	−
1:2500	+ + + +	+ + +	−
1:3000	+ + + +	+ + +	−
1:3500	+ + +	+ +	−
1:4000	+ + +	−	−
1:4500	+ +	−	−
1:5000	+	−	−

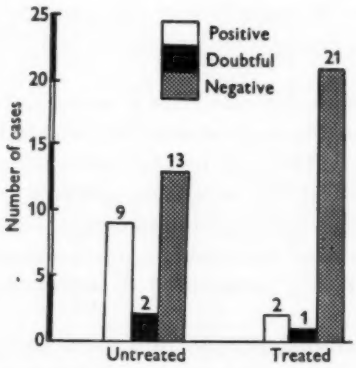

Fig. 2. C.F.T. with acetone precipitated fraction tested against sera of twenty-four treated and twenty-four untreated cases of bilharziasis.

(3) The results of the titration of extract VII with the pooled positive serum are shown in Table 2 and the results of C.F.T.'s with the patients' sera in Fig. 2. The supernatant after precipitation of this fraction was tested and found not to contain complement-fixing antigens.

These results, although similar, are not directly comparable with those obtained by means of the analogous extract V as the tests were not performed simultaneously. Not always the same sera gave positive results with both fractions.

B. Fasciola hepatica *worm extracts*

Extracts I–IV were examined. Complement-fixing activity was evident in extracts II and V only, but the titres of these were low and the effective dilution (1:100) too anti-complementary, for use in the test.

C. *Immunization of rabbits*

The sera of all rabbits gave negative C.F.T.'s with each of the extracts I, II and VI before the course of injections and afterwards, only that rabbit immunized against extract I reacted positively and then only to its homologous antigen. Each rabbit, on being skin tested with each of the three antigens, gave negative reactions.

DISCUSSION

It has been shown that the principle in *S. mansoni* worms which acts as antigen in the C.T.F. for bilharziasis can be extracted by Coca's solution and by absolute alcohol. The solubility of the antigen in aqueous as well as in lipid solvents suggests that either it is a lipoprotein complex or that two or more active substances of different chemical nature are present. The lipid component of the antigen from *S. mansoni* worms is soluble in absolute alcohol but not in acetone, and is precipitated by the latter. As the extracts II and V proved equally potent, ether has no influence on the active principle. The extract in Coca's solution contains polysaccharide, as demonstrated by the anthrone test (Morris, 1948), but it has not been established whether the carbohydrate plays any part in the C.F.T. However, a polysaccharide extract obtained by Fuller's method (1938) showed no antigenic properties.

In interpreting the results differentiation must be made between specificity and sensitivity of the antigens. The lipid extracts II, V and VII were more sensitive but less specific than extract I. This may well be due to the presence in the former of alcohol-soluble, acetone-insoluble, sensitizing agents, e.g. of the lecithin type. It seems that certain acetone-soluble substances depress the specificity of the antigen since their removal increased the accuracy of the diagnosis from 13 and 4% (extract II) to 42 and 13% (extract V) in untreated and treated patients respectively.

It is interesting to recall that Fairley (1919, 1925, 1927), working with antigens prepared from the livers of infected snails, concluded that the active principle was a pure lipid, while LeBas (1922) proposed the view that it was a protein. It should, however, be borne in mind that these authors did not work with adult worms.

Results obtained with *F. hepatica* extracts, though disappointing, confirm the trend seen with *S. mansoni* extracts. Thus extracts II and V showed activity but only at the lowest dilution tested (1:100) while the Coca extract was devoid of activity even at this level.

Whatever the nature of the antigen may be, it appears that for use in clinical diagnosis, an extract of *S. mansoni* worms in Coca's solution is the best, while extracts of *F. hepatica* worms are unsuitable for this purpose.

SUMMARY

1. Different extracts of adult worms of *S. mansoni* and *F. hepatica* have been examined in the C.F.T. for bilharziasis. Extracts of *S. mansoni* worms in Coca's solution, alcohol-ether and absolute alcohol after acetone extraction possess high antigenic activity, the first being the most specific but less sensitive than the others. Extracts in Coca's solution of alcohol-ether insoluble residues still showed slight activity while acetone and polysaccharide (formamide) extracts showed none.

2. Using the Coca's extract of *S. mansoni* worms, the C.F.T. was positive in 83 % of sera of untreated patients and 58 % of treated. The figures using the alcohol-ether extract were 13 and 4% respectively and, for the alcoholic extract of acetone insoluble residue, 42 and 13%.

3. Extracts of *F. hepatica* worms showed the same general trend, but even in the two extracts to show activity (alcohol-ether and alcohol extract of acetone insoluble material) the titre was too low for use in the test.

4. Of rabbits immunized with three different fractions of *S. mansoni* worms only that given the Coca extract showed complement-fixing antibodies and then only with the homologous antigen. Skin tests on the rabbits were negative to each of the antigens used.

5. The nature of the antigen in the C.F.T. is discussed, and it is suggested that the active portion is lipo-protein in nature or more than one substance is involved. For use in clinical diagnosis, an extract of *S. mansoni* worms in Coca's solution is recommended.

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PARAMPHISTOMUM SUKARI N.SP. FROM KENYA CATTLE AND ITS INTERMEDIATE HOST

By J. A. DINNIK

East African Veterinary Research Organization, Muguga, Kenya

(With 7 Figures in the Text)

Amongst several species of paramphistomes collected from cattle in the Kenya Highlands a species was found which shows a number of features which are different from those of species hitherto described. This species was first discovered on the farm of Sukari Ltd., situated near Nairobi, Kenya, where forty-one out of fifty-four cattle examined were found to be infected with this fluke. Specimens collected from these cattle were used for the following description of the species for which the name *Paramphistomum sukari* is proposed.

Paramphistomum sukari n.sp.

Morphology of the species. The conical-shaped body of mature specimens is from 6 to 10 mm. long and from 1·6 to 2·5 mm. wide, if measured dorso-ventrally at the level of the testes (Fig. 1).

The acetabulum is from 1·3 to 1·6 mm. in diameter. The ratio of the diameter of the acetabulum to the body length varies from 1:4 to 1:6. The dorsal and ventral halves of the external circular acetabulum musculature, as seen in median sagittal sections, are identical, and both are separated from the internal acetabulum tissue by an obliquely running muscle band, although this band is not strongly developed. The radial muscle fibres of the acetabulum are moderately coarse and run individually. Table 1 shows the number of units in various circular muscle series in fifteen specimens of *P. sukari*:

Table 1. *The number of units in circular muscle series of* Paramphistomum sukari *n.sp.*

Muscle series	Number of units														
Dorsal exterior	15	13	12	13	15	16	15	14	15	14	15	13	15	15	15
Dorsal interior	36	31	31	34	33	32	31	35	33	34	31	32	34	33	39
Ventral interior	38	31	32	29	34	33	37	36	35	30	32	40	35	31	36
Ventral exterior	14	13	14	13	15	14	14	14	11	13	14	13	15	15	14

The pharynx is of the *Paramphistomum* type. The internal circular muscle layer consists of small closely packed units. The internal longitudinal layer occupies about one-third of the thickness of the pharynx and is indistinctly delimited externally. The external longitudinal layer is strong, narrow, and sharply delimited internally. The external circular layer is well developed only in the posterior half of the pharynx, where its units consist of several somewhat separated muscle fibres. The radial layer consists of bundles of fibres running between the units of

the external circular layer. The basal circular layer is composed of strong muscle units lying in two rows. The middle circular layer of muscle, posterior pharyngeal sphincter, and lip sphincter are lacking.

The pharynx is from 0·55 to 0·80 mm. long, which gives a ratio of from 1:10·42 to 1:12·5 in relation to the length of the body and from 1:2·0 to 1:2·7 in relation to the external diameter of the acetabulum.

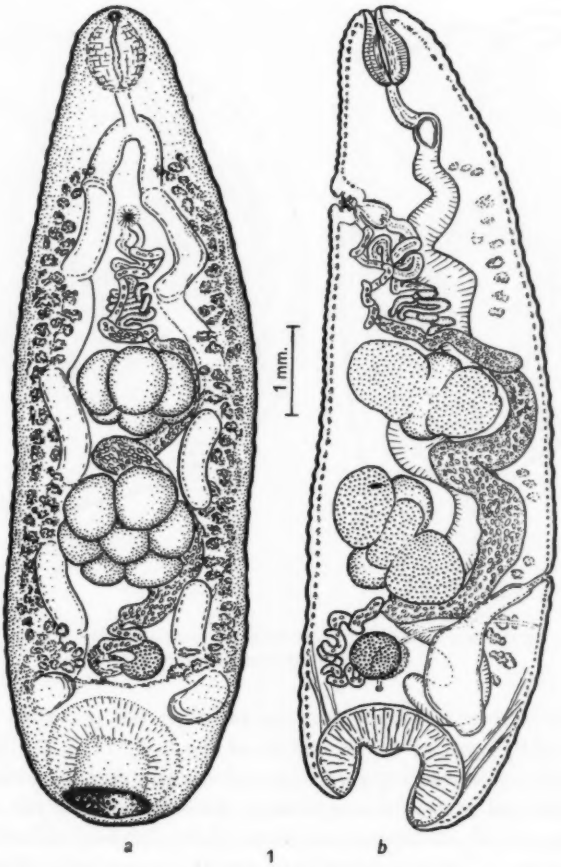


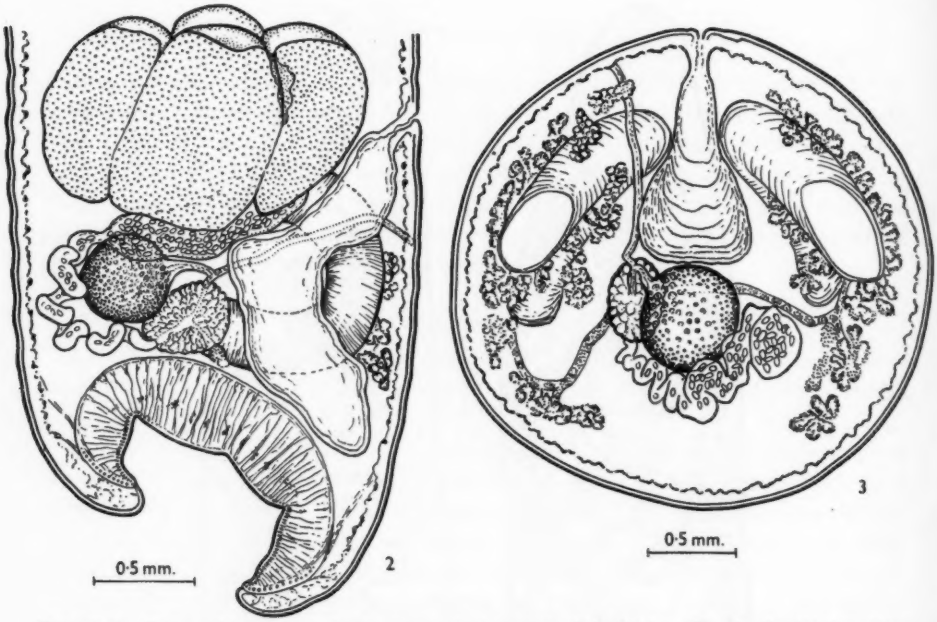
Fig. 1. *Paramphistomum sukari* n.sp. (*a*) ventral view; (*b*) sagittal section of the same specimen.

The oesophagus is from 0·55 to 0·70 mm. long and it is more or less curved dorsally towards its junction with the gut caeca. Each gut caecum has three main convolutions, the degree of which depends upon the state of contraction of the body. Anterior to the acetabulum the terminal parts of the caeca turn towards the ventral surface and their blind ends are directed ventrally.

The excretory bladder lies dorsal to the acetabulum and extends anteriorly to the level of the posterior border of the hinder testis. A short duct runs from the

bladder to the excretory pore, which opens in the mid-line of the body at about the level of posterior border of the hinder testis.

The testes are situated one behind the other. Both are lobed deeply, each having from five to eight lobes. In sagittal sections the testes appear to be elongated dorso-ventrally and both are of approximately equal size, measuring from 1·2 to 2·0 mm. dorso-ventrally and from 0·6 to 1·3 mm. antero-posteriorly.



Figs. 2, 3. *Paramphistomum sukari* n.sp. posterior part of the body. Fig. 2. Sagittal section. Fig. 3. Tranverse section.

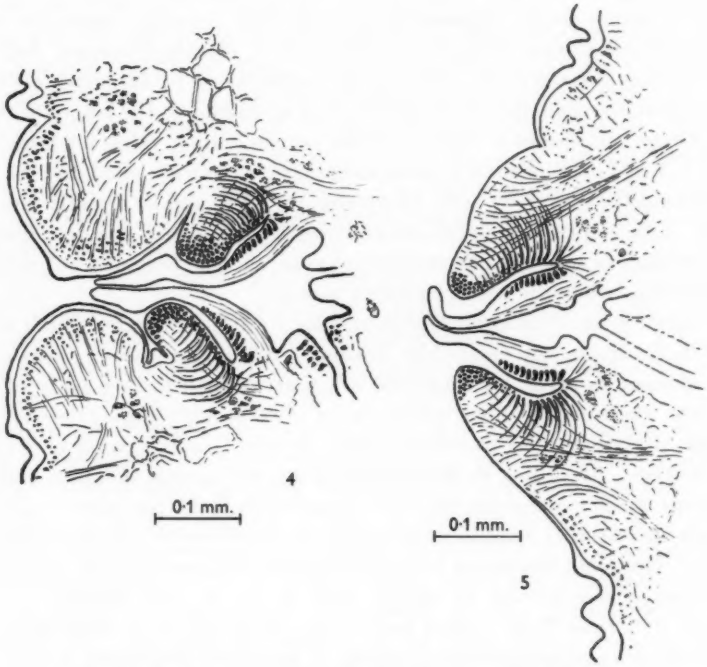
The ovary is oval or spherical and lies between the posterior testis and the acetabulum, slightly ventral to the middle of the body (Fig. 2). Mehlis' gland is close to the ovary, on the left or right side and usually slightly posterior to it. The gland is compact and more or less spherical. Measurements of the ovary are from 0·27 × 0·46 mm. to 0·56 × 0·73 mm. and those of Mehlis gland from 0·25 × 0·35 mm. to 0·45 × 0·62 mm. A short oviduct connects the ovary with Mehlis' gland and outside this gland it is joined by Laurer's canal which runs dorsally from this point, crosses the excretory bladder and opens posterior to the excretory pore. The opening is located 0·25–0·65 mm. behind the excretory pore and somewhat to the side (Fig. 3).

Clusters of vitelline gland extend from the level of the junction of the gut caeca to the acetabulum.

The genital pore is situated 0·4–1·0 mm. posterior to the level of the gut junction and lies 1·3 mm. from the anterior end of the fluke.

The genital atrium is provided with a genital sphincter and sphincter papillae

(Fig. 4). The genital sphincter is strongly developed and clearly delimited from the surrounding tissue. The radial musculature of the genital atrium is also well developed and its fibres are evenly curved. In most specimens examined the papilla was found partially or entirely extended, but in some specimens it was greatly retracted. The sphincter papillae is fairly strong and appears as a large area at the base of the papilla. Outside the wall of the genital atrium is a small ventral atrium which is invisible when the genital atrium is protruded (Fig. 5).



Figs. 4, 5. *Paramphistomum sukari* n.sp.; genital atrium.

The pars prostatica is 0·3–0·4 mm. long. The pars musculosa is very long and strongly coiled. The width of the proximal part of the pars musculosa is from 0·13 to 0·20 mm. The closely coiled vesicula seminalis is situated posterior to the pars musculosa.

The eggs are 0·137–0·165 mm. × 0·071–0·092 mm.

Geographical distribution. Kenya, East Africa. The species has been found in cattle in the vicinity of Nairobi, Nanyuki, Nakuru, and in cattle from the Masai District.

Host. Bos taurus.

Habitat. Reticulum.

Näsmarck (1937), who had ample sectioned material to study, came to the conclusion that the structure of the acetabulum, pharynx, and genital atrium may be used for the systematic study of paramphistomes. Using these criteria he has

confirmed the validity of many species which Maplestone (1923) regarded as synonyms of *P. cervi* (Zeder), described some new species and subdivided the old genus *Paramphistomum* Fischoeder, 1901 into eight genera.

Although in the acetabulum of the new species the dorsal and ventral halves of the external circular musculature are identical, the species cannot be placed in the genus *Calicophoron* Näsmark, 1937, since the distinctive features of the latter also include the genital atrium (which is of peculiar type) and the long and strongly developed pars prostatica.

The new species in its appearance and internal anatomy resembles *Paramphistomum microbothrium* Fischoeder, 1901, and *P. clavula* Näsmark, 1937, more closely than any species of the other genera established by Näsmark (1937). Its body is conical, the diameter of the acetabulum in relation to the length of the body varies between the values 1:4 and 1:6 (the corresponding value for *Calicophoron* are from 1:3 to 1:3,4), Laurer's canal crosses the excretory canal and its pore is located behind the excretory pore. The testes are lobed and lie directly one behind the other. Like the two *Paramphistomum* species mentioned, the new species has a genital atrium of modified *Microbothrium* type and the long and strongly coiled pars musculosa, while the pars prostatica is short.

The original genus *Paramphistomum* retains a group of species which were not included in the new genera created by Näsmark (1937). The characteristics common to these species, as given by Näsmark, are that they all have a body of conical shape, that their acetabulum which is of *Paramphistomum* type is placed terminally, and that their excretory canal crosses Laurer's canal and opens in front of its pore. According to this definition the new species should not be placed in the genus *Paramphistomum* since its acetabulum lacks the second part of the dorsal external circular muscle units which is characteristic of the *Paramphistomum* type. However, the genus *Paramphistomum* Fischoeder, 1901, still contains morphologically two groups of species such as (1) *P. cervi* (Zeder), *P. gracile* Fisch., and *P. liorchis* Fisch., which have a very short pars musculosa and a genital atrium without a genital sphincter; and (2) *P. microbothrium* Fisch. and *P. clavula* Näsmark, which possess a long strongly developed and coiled pars musculosa and genital atrium with strongly developed genital sphincter. It is apparent that the new species belongs to the same group as *P. microbothrium* and *P. clavula*, and consequently it has to be placed in the genus *Paramphistomum*.

P. sukari n.sp. is easily distinguished from both *P. microbothrium* Fisch. and *P. clavula* Näsmark by its testes, which have only from five to eight lobes each and by the structure of the acetabulum, in which the second part of the dorsal external circular layer of muscle units is lacking.

DEVELOPMENT IN AN INTERMEDIATE HOST

An examination of the snails *Bulinus alluaudi* (Dautzenberg), *Limnea caillaudi* (Bgt.), and *Biomphalaria pfeifferi* (Krauss), collected from ditches in the cattle pastures on the farm of Sukari Ltd., showed that *Bulinus alluaudi* was infected with larval stages of *Paramphistomum microbothrium* Fisch. while *Biomphalaria pfeifferi* contained rediae and developing cercariae of another paramphistome.

Infected specimens of *B. pfeifferei* were particularly numerous in those sections of the ditches which the cattle used as watering places. Where cattle droppings were scattered abundantly near water, the rate of infection of the snails reached up to 35%, and these snails were heavily infested with numerous radiae and developing cercariae. Eight infected molluscs collected from one such watering place emitted 750 cercariae the first day and 584 cercariae the following day, when they were placed in direct sunlight. These cercariae are heavily pigmented and show two dark eyespots near the oral sucker. A profuse supply of cystogenous cells, together with the pigmentation, obscure the internal structure of the cercariae. The body is about 0·25 mm. long and 0·17 mm. broad, the tail being about twice the length of the body. After a free swimming period of a quarter of an hour the cercariae encysted on the sides of the glass container. The cysts are much smaller than those of *P. microbothrium*, almost spherical in shape, round in surface view, and dome-shaped when viewed laterally. The slightly expanded base attaches the cyst to substratum. The diameter of the cysts varied from 0·127 to 0·138 mm. and their height from 0·111 to 0·121 mm.

About 300 encysted cercariae obtained from *Biomphalaria pfeifferi* were fed to each of two goats. One of these goats was slaughtered 92 days later and twenty-three immature specimens of *Paramphistomum sukari*, from 2·0 to 2·5 mm. long, were found in the reticulum. The second goat sacrificed on the 150th day was found to be devoid of flukes.

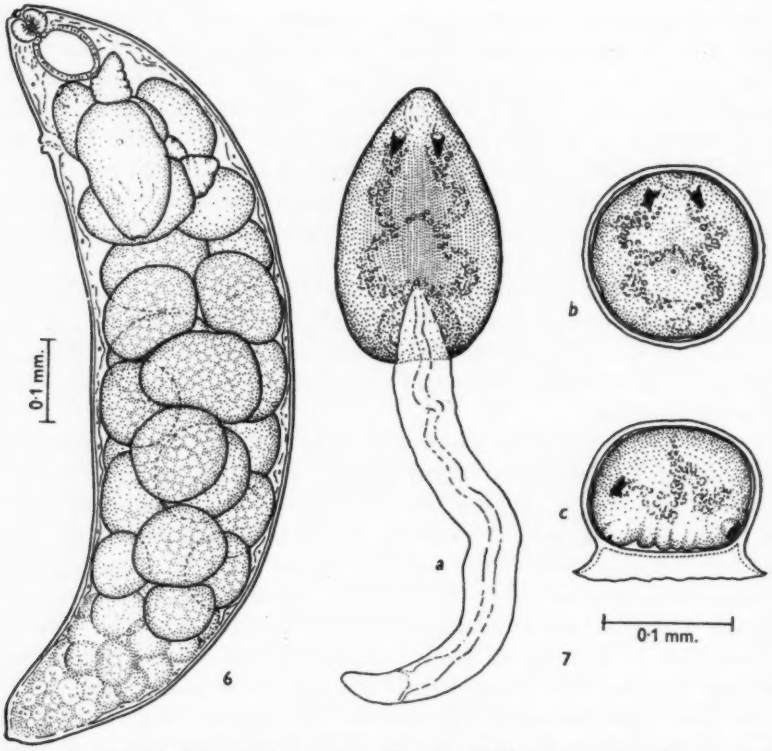
To confirm the finding of an intermediate host of *P. sukari* three attempts were made to infect the laboratory-reared specimens of *Biomphalaria pfeifferi* with miracidia of this species. Eggs of *Paramphistomum sukari* were obtained from adult flukes collected from the reticula of cattle slaughtered at the farm of Sukari Ltd. The eggs hatched into miracidia on the 15th day, when kept in water at a temperature of about 27° C. For infection, snails were placed in a glass basin containing water to which numerous freshly hatched miracidia were added. After being left in the basin for about 4 hr. the snails were transferred to aquaria where they were fed with cooked lettuce and kept under observation.

In the first experiment thirty-seven snails were exposed to miracidia. Most of these snails died within 20 days, and on the 21st day only four snails remained alive. One of them was dissected and examined and was found to contain three sporocysts and many rediae of different sizes. In the sporocysts five, seven and eight young rediae respectively and some embryo balls were seen. The smallest redia, found in the liver of the snail, had apparently just escaped from a sporocyst, and were 0·23 × 0·06 mm. The mature rediae found in the snail were up to 1·5 × 0·2 mm. The diameter of the pharynx varied from 0·036 to 0·046 mm., and the gut reached 0·08 mm. in length. The body cavites of mature rediae were filled with 35 to 50 embryo balls. The anterior part of the body cavity of some mature rediae showed two to four developing cercariae and only one very young developing cercaria was found free in the liver of the snail. The three snails which remained in the aquarium died in the course of the following few days, thus terminating the experiment.

In the second experiment fifty-three specimens of *Biomphalari pfeifferi* were

exposed to miracidia. With the exception of two all the snails died shortly after exposure. The two remaining snails dissected 30 days later contained no larval stages of flukes.

In the third experiment 217 snails were exposed to miracidia of *Paramphistomum sukari*. Although the mortality rate of the snails was again high, some survived long enough for the completion of the larval development of *P. sukari*. Two



Figs. 6, 7. *Paramphistomum sukari* n.sp. Fig. 6. Redia. Fig. 7*a*. Mature cercaria. Fig. 7*b* and *c*. Encysted cercariae.

sporocysts and twenty rediae were recovered from the snail of this series which was dissected 18 days after exposure to miracidia. One sporocyst was inadvertently destroyed during dissection of the snail and some very young rediae—the number of which was not determined—squeezed out. The other sporocyst (0·40 × 0·21 mm.) contained twelve young rediae and ten embryo balls. In one free redia a daughter redia and two redial embryos were clearly recognizable while the remaining young rediae of different sizes contained only embryo balls.

The second snail, dissected on the 24th day after exposure to miracidia, showed two sporocysts and many rediae. In the body cavities of some of the rediae daughter rediae and the redial embryos were seen. No developing cercariae were found in the snail.

The snail dissected on the 30th day was found to contain rediae of different sizes. The mature rediae (1–1·8 mm. × 0·18–0·30 mm.) contained from one to five developing cercariae and as many as up to fifty embryo balls. The pharynx was from 0·036 to 0·050 mm. in diameter and the gut caeca 0·12 mm. long (Fig. 6). Numerous free cercariae in different stages of development infested the liver of the snail. The development of many of them had proceeded to the stage when the body had become heavily pigmented and the tail fully developed. Some rediae found in this snail were of dirty brown colour, almost opaque and contained from one to three young, well-developed daughter rediae, together with a few embryo balls. It was apparent that these rediae were old and approaching the end of their productivity and life.

The first emergence of the cercariae was observed in the aquarium 35 days after exposure of the snails to miracidia. On the 29th day, when ten snails which had survived up to this time were placed in the light of a strong electric bulb to stimulate the emergence of cercariae, only three snails shed cercariae. The cercariae swam actively in the water for a short time; within 20 min. they all encysted on the wall of the glass container. In general appearance, shape and measurements the cercariae and cysts were identical with those obtained from the naturally infected snails collected from the ditches in the cattle pastures at the farm of Sukari Ltd. (Fig. 7).

The three infected snails died within a month from the day of the first shedding of cercariae. The remaining seven snails were dissected and found to be not infected.

SUMMARY

Paramphistomumi sukari n.sp. from the reticulum of cattle in the Kenya Highlands (East Africa) is described. An intermediate snail host of *P. sukari* was shown to be *Biophalaria pfeifferi* (Krauss).

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AMOEBAE FOUND IN THE INTESTINE OF LIZARDS FROM THE SUDAN

By R. A. NEAL

Wellcome Laboratories of Tropical Medicine, London

(With 9 Figures in the Text)

Very little information is available concerning the group of species of *Entamoeba* which form 8-nucleate cysts, apart from *E. coli* of man and *E. muris* of rodents. As lizards have been reported to harbour amoebae of this type (Wenyon, 1921; etc.). the opportunity was taken while working in the Sudan to search for this amoeba and to isolate it *in vitro* so as to be able to study it in detail at a more convenient time. Although cultivation of this amoeba failed, it was thought worth while to record briefly the following observations.

MATERIAL AND METHODS

The lizards examined were *Mabuya quinquetaeniata* (skink), *Tarentola annularis* (gecko), *Chalcides ocellatus* (skink)—all caught at Khartoum, mainly around the veterinary compound—and *Mabuya striata* (skink). The specimens of the last-named lizard were caught at Malakal, which is situated on the White Nile, 450 miles south of Khartoum. The lizards were killed by chloroform, and smears from the large intestine were prepared with saline solution and Lugol's iodine solution, and examined immediately after death of the animal. Where amoebae were observed, smears were fixed in Bouin's solution, transferred to 70 % ethanol, and brought to England where they were stained. Stains used were Heidenhain's iron haematoxylin with or without eosin counter-staining and Dobell's phosphotungstic haematoxylin.

Intestinal contents containing the amoebae were also inoculated into culture media, *hsm* + *S* or *hs* + *S* [diluted horse-serum plus autolysed yeast extract (Jones, 1946) or diluted horse-serum; both media supplemented with rice starch] and left at room temperature (about 50–90° F.). The amoebae generally survived in the primary culture for several days, and in some instances even multiplied, but always died out when subcultured. In one culture from *M. quinquetaeniata*, the amoebae multiplied and encysted in large numbers but the amoebae would not grow when subcultured nor would the cysts hatch when inoculated into fresh medium. More determined attempts at cultivating would possibly meet with more success but only a limited amount of time was available for the present work.

The figures were drawn with the aid of a camera lucida at a magnification of 2000.

OBSERVATIONS

Examination of the prepared slides revealed the presence of two distinct amoebae, an *Entamoeba* forming octanucleate cysts and an '*Amoeba*' with uninucleate cysts. These two organisms are described separately.

(*a*) *Morphology of the* Entamoeba

This amoeba was found in *Mabuya quinquetaeniata* (1 positive out of 45 examined), *M. striata* (6 positive out of 49 examined) and *Tarentola annularis* (1 positive out of 12 examined).

When alive the amoeba progressed by a slug-like movement, the nucleus being invisible or only faintly visible. A contractile vacuole was not observed. The food vacuoles contained bacteria and intestinal debris.

Fixed and stained amoebae measured, when rounded, from 11 to 20 μ in diameter, with the nucleus measuring about 4 μ in diameter. The nuclear structure was very similar to that of *Entamoeba coli* of man, the karyosome being an irregularly rounded structure generally eccentrically situated (Figs. 1 and 2);* the peripheral chromatin was arranged on a thin nuclear membrane as discrete granules. Between the karyosome and nuclear membrane a few granules, staining much less intensely than the karyosome or the peripheral chromatin, were observed.

The cysts (Figs. 3 and 4) were spherical in shape, though irregular specimens were sometimes observed. Mature 8-nucleate cysts were measured, after colouring by Lugol's iodine, from *Mabuya striata* and from a primary culture from *M. quinquetaeniata*. These data are shown in Table 1.

Table 1. *The size of mature cysts of the* Entamoeba

Origin of cysts	No. measured	Range (μ)	Mean $\pm$ S.E.(μ)
M. striata	100	12·9–21·0	17·11 $\pm$ 0·17
Culture	100	12·9–25·8	18·85 $\pm$ 0·19

These measurements when plotted graphically gave unimodal polygons with modes at 17·8 μ in both cases.

Chromatoid bodies were generally absent in the mature cysts, but when present were acicular in shape (Fig. 4). The structure of the nuclei was similar to that in the amoebae.

Binucleate cysts, not illustrated, developed a large glycogen vacuole similar to those of *Entamoeba coli* of man, but other stages of encystment were not seen.

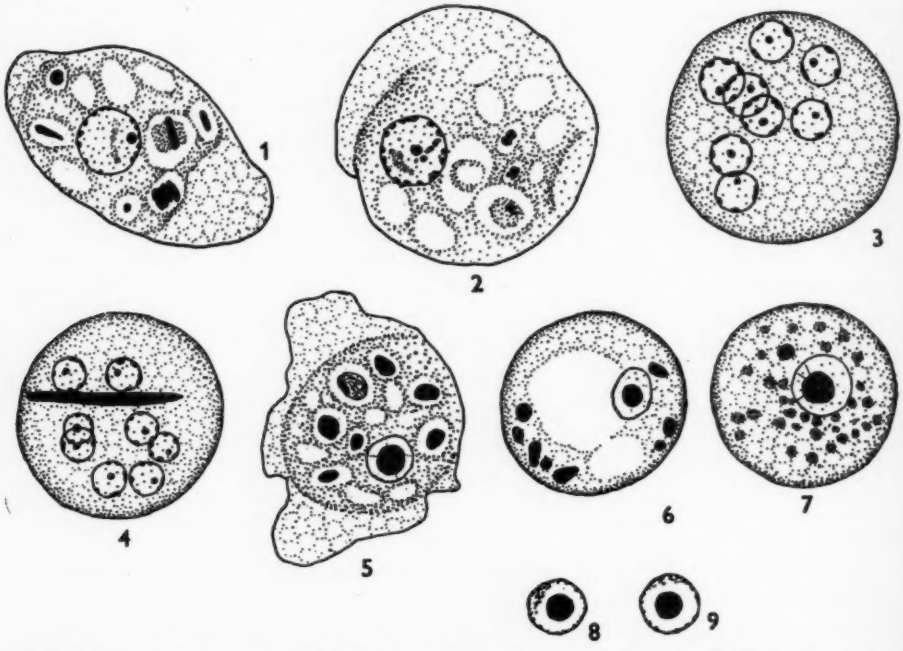
(*b*) *Morphology of the* 'Amoeba'

This organism was less common than the *Entamoeba*, being found in 2 of the 12 specimens of *Tarentola annularis* examined and not in the other species of lizard. One gecko was found infected with amoebae in fairly large numbers whilst in the other infected gecko, amoebae and cysts were seen. The latter animal was also infected with the *Entamoeba*.

The living amoebae were active organisms, their movement being of the slug-like type; the nucleus was faintly visible in the endoplasm. Contractile vacuoles were not observed.

* Careful focusing showed that the karyosome of the amoeba illustrated in Fig. 2 was actually eccentric, but the position from which the nucleus was seen is such that it appears almost centrally placed.

When fixed and stained they measured from 15 to 20 μ in diameter and the contents of the food vacuoles showed they ingested bacteria and intestinal debris. Although the '*Amoeba*' resembled the *Entamoeba* in size, the nuclear structure was quite different. The nuclear membrane was very thin, often only just visible, without a lining of chromatin, while at the centre of the nucleus was a very large karyosome (about 2 μ in diameter, Fig. 5). The karyosome was a homogeneous



Figs. 1–7. Amoebae found in *Mabuya striata* and *Tarentola annularis*. × 2000 (2 mm. = 1 μ).

Figs. 1–7 fixed in Bouin's solution and stained with Heidenhain's haematoxylin, Figs. 1 and 2 counterstained with eosin. Fig. 1. Typical *Entamoeba* showing nucleus with very eccentric karyosome; from *M. striata*. Fig. 2. *Entamoeba* with an apparently centrally placed karyosome; from *M. striata*. Fig. 3. Typical *Entamoeba* cyst; from *M. striata*. Fig. 4. *Entamoeba* cyst with acicular chromatoid body; from *M. striata*. Fig. 5. Typical '*Amoeba*'; from *T. annularis*. Fig. 6. Cyst of '*Amoeba*' showing vacuole and chromatoid bodies; from *T. annularis*. Fig. 7. Cyst of '*Amoeba*' showing one chromatoid body and numerous lightly staining granules; from *T. annularis*.

Figs. 8 and 9. Nuclei of Dobell's *Entamoeba* drawn from the original preparations, from *Lacerta muralis*. Fixed in sublimate-alcohol, stained with Delafield's haematoxylin.

structure, even with drastic de-staining no discrete granules could be seen but vacuolation was sometimes observed. In the area between the karyosome and nuclear membrane faint threads of chromatin radiated from the karyosome to the nuclear membrane, otherwise that part of the nucleus was devoid of chromatin. The nucleus measured about 4 μ in diameter.

Cysts of this amoebae were observed in one animal (Figs. 6 and 7). They were always uninucleate, spherical in shape and measured from 11 to 17 μ in diameter

(average of 20 cysts, 12·6 μ). An empty vacuole was seen in stained preparations of some cysts (Fig. 6) which may have been originally filled with glycogen as in the case of the *Entamoeba*, but unfortunately this point was not investigated in iodine stained preparations.

The vacuole is not due to artifacts since the cyst wall is perfectly regular, the cytoplasm has not shrunken from the cyst wall and the nucleus presents a normal appearance. The cysts without the vacuole were generally smaller than those with this structure, though they were occasionally of the same size (compare Figs. 6 and 7).

Large irregularly rounded granules were also present in the cytoplasm of the cysts; these were stained black with the iron haematoxylin stain while with the phosphotungstic haematoxylin they were less intensely coloured than the karyosome of the nucleus. By analogy with similar granules in *Entamoeba* cysts these could be called chromatoid bodies. The cystic cytoplasm was also frequently filled with granules which stained lighter than the bodies just mentioned and were also slightly smaller with less well-defined edges (Fig. 7).

(*c*) *Other Protozoa*

As noted above, the Protozoa principally studied were the amoebae, but the presence of other types of protozoan parasites was noted as follows: *Mabuya quinquetaeniata—Proteromonas* (= *Prowazekella*) *lacertae*, *Chilomastix* sp., *Eimeria* sp.; *Mabuya striata—Proteromonas lacertae*, *Chilomastix* sp.; *Chalcides ocellatus—Proteromonas lacertae*; *Tarentola annularis—Proteromonas lacertae*, *Nyctotherus* sp.

DISCUSSION

The morphology of the *Entamoeba* is very similar to that of *E. coli* of man. The arrangement of the peripheral chromatin however is different, as in the lizard *Entamoeba* the granules are distinctly separate from each other, while in the human species the granules are generally much closer, often appearing in optical section as a continuous band. The amount of material of the lizard *Entamoeba* is insufficient to state definitely whether this difference is always present. It has not been noted by other workers in similar amoeba from lizards.

The following lizards have also been reported to be infected with an amoeba morphologically similar to the present organism: *Lacerta agilis* and *Agama stellio* from Egypt (Wenyon, 1921); *Hemidactylus flaviviridis* from India (Knowles & Das Gupta, 1935); *Xantusia vigilis*, *Sceloporus occidentalis bi-seriatus*, *S. magister*, *S. graciosus vandenbergianus*, *Uta stansburiana*, *Dipsosaurus dorsalis*, *Callisaurus ventralis* and *Sauromalus obesus* caught in California, U.S.A. (Wood, 1935); and the Mexican lizard *Sceloporus clarkii* (Hegner & Hewitt, 1940). The *Entamoeba* has been given various names, *E. flaviviridis* (Knowles & Das Gupta), *E. lacerticoli* (Wood) and *E. ctenosaurus* (Hegner & Hewitt).

It should be noted that Wenyon records the presence of smaller entamoebae, measuring about 10 μ or less in diameter. It is possible that these amoebae may belong to a different species, similar perhaps to that described by Franchini (1921) forming 4-nucleate cysts, or to the very similar *E. cuantlae* of Hegner & Hewitt (1940).

In view of the close morphological resemblance of the *Entamoeba* described in the present work with those described previously, it is proposed to refer the present *Entamoeba* to the species *E. flaviviridis* Knowles & Das Gupta; this name having priority over those given by the other authors.

Dobell (1914) described two amoebae from *Lacerta muralis*, one which formed uninucleate cysts he called *Amoeba lacertae* and is similar to the '*Amoeba*' described above, the other he considered to be an *Entamoeba*. An unusual feature of the latter organism is the relatively large karyosome, therefore, in order to elucidate its relationships with other lizard amoebae, I have examined Dobell's original preparations.

Dobell's *Entamoeba* is distinguished at once from *Amoeba lacertae*—the latter are considerably more abundant—by its larger size. In preparations stained by Heidenhain's haematoxylin this is the only distinguishing feature as the granules of the peripheral chromatin are only faintly visible or invisible. The karyosome, however, stains intensely in the normal manner. The peripheral layer is better stained by Delafield's haematoxylin and appears as a number of very small granules lining the nuclear membrane (Figs. 8 and 9) but not as closely applied to the membrane as in the *Entamoeba* I found in Sudanese lizards. The peripheral layer may be composed of more than one layer of granules. The karyosome appears as a large homogeneous structure when stained by either technique. Cysts were not found in Dobell's preparations, nor were entamoebae of this type observed in the Sudanese lizards.

The nuclear characters of Dobell's *Entamoeba* differ from the typical *Entamoeba* structure by the diffuse arrangement of the peripheral chromatin granules and by the relatively large size of the karyosome. On the basis of these differences, it seems uncertain whether the organism should be classified as an *Entamoeba*.

The morphology of the '*Amoeba*' described above agrees very closely with that of an amoeba from *Sceloporus undulatus* (McFall, 1926). It differs in only two respects: (*a*) the nucleus of McFall's amoeba was often diamond-shaped, whereas mine was spherical, and (*b*) I observed vacuolation of the karyosome whereas McFall did not. It also does not seem likely that McFall's description of nuclear division is correct as it does not agree with recent work on this subject in amoebae.

The *Amoeba* described by Dobell (1914) and by Wood (1935) was similar in structure to the amoeba described in the present work differing from the latter only in the smaller size.

Very late in the course of this investigation it was realized that the cysts of the '*Amoeba*' could be confused with the intestinal stage of a primitive fungus which has been described from lizards, namely *Basidobolus lacertae*. By this time only fixed material was available for the differentiation of these two organisms. After studying the description of *B. lacertae* given by Loewenthal (1903), the only distinguishing morphological features seem to be the presence of chromatoid bodies in the '*Amoeba*' cysts and the smaller size of the latter (average diameter 12·6 μ) compared with the forms of *B. lacertae* (average diameter 17 μ). They otherwise appear to be very similar morphologically. Other evidence which supports the belief that these bodies actually are a cystic stage in the life history of the '*Amoeba*'

is that the cysts were found only in company with the amoeboid phase and they were not observed, unaccompanied by the amoeboid phase, in any other lizard.

The classification of the '*Amoeba*' described in the present work presents great difficulty owing to the chaos existing in the classification of these amoebae at the present time. The main criterion is the method of nuclear division (Singh, 1950 and others), but in spite of much searching it was not observed in the present '*Amoeba*'. McFall referred his amoeba to the genus *Endolimax*, however Reichenow, in his revision of Doflein's text-book (1928), placed it in the genus *Vahlkampfia*. In the light of recent work *Vahlkampfia* has been split into several other genera, but the available data do not provide sufficient criteria for the generic position of the present '*Amoeba*'.

SUMMARY

1. The morphology of an *Entamoeba* from the Sudanese lizards *Mabuya quinquetaeniata*, *M. striata* and *Tarentola annularis* is described. It was similar to *Entamoeba coli* of man.

2. It is referred to the species *E. flaviviridis*, Knowles & Das Gupta (1935).

3. An '*Amoeba*' was observed in *Tarentola annularis* which was similar in size to the *Entamoeba*. The nuclear structure, however, was distinctive, with a very large karyosome and no peripheral chromatin. The cysts were uninucleate.

4. It is very similar to the organism described as *Vahlkampfia reynoldsi* by McFall (1926).

I should like to thank Mr J. T. R. Evans, Director, Sudan Veterinary Service, for facilities to carry out this work, and Mr J. C. Battersby, British Museum (Nat. Hist.), for identification of *Mabuya striata*.

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OBSERVATIONS ON THE FOOD AND THE GUT PIGMENT OF THE POLYOPISTHOCOTYLEA (TREMATODA: MONOGENEA)

By J. LLEWELLYN, Ph.D.

Department of Zoology and Comparative Physiology, University of Birmingham

(With Plate XV)

CONTENTS

		PAGE
I.	Introduction	428
II.	Material	430
III.	Morphology and histology of the alimentary canal and its contents	430
IV.	The nature of the gut contents	431
V.	Comparison with the monopisthocotylea	434
VI.	Discussion	434
VII.	Summary	436
	References	436

I. INTRODUCTION

The Polyopisthocotylea constitute one of the two suborders of monogenetic trematodes, and contain (Sproston, 1946) three super-families: the Avielloidea including a single species from Lake Baikal, the Polystomatoidea (two families, seventy-six species, including *Polystoma integerrimum* from the bladder of the frog), and the Diclidophoroidea (six families, 188 species, including *Diclidophora merlangi* from the gills of the whiting). The literature on the group contains but few references to the nature of the food of these trematodes, and some of the observations that have been made are conflicting.

Goto (1895, p. 180) stated that the majority of the species of Monogenea that he had examined lived on the slime of their hosts, but that some were able also to extract blood.

Gallien (1935), in the course of extensive studies upon *Polystoma integerrimum*, made frequent references to the blood-feeding habits of both adults and larvae in the bladder of the frog, and of neotenics on the gills of tadpoles; in fact, he went so far as to attribute the neotenic development to the influence of hormones present in the blood ingested from the host. Gallien's identification of the gut contents as blood appears to have depended solely upon their colour except in one case (1935, p. 20) when erythrocytes were observed. In the case of those larvae which commenced feeding whilst they were on the gills and which were thus embarking upon a neotenic development, Gallien was of the opinion that only blood plasma which had filtered through the walls of the gills was absorbed, since he did not observe any erythrocytes in the gut of these larvae. He further estimated that actual perforation of the gill tissue by the feeding activities of the large numbers (about 100) of larvae infesting a single tadpole host under experimental conditions would in any case have proved fatal.

Bonham & Guberlet (1937) observed that specimens of *Microcotyle sebastis*, recently removed from their hosts, vomited ingested red blood cells into the water, but Dawes (1946, p. 525), commenting on the habits of *Microcotyle*, stated that it was not at all likely that it fed upon blood sucked from the capillaries of the gills. The latter author (1940, 1946, p. 531) stated that he had experienced much difficulty in attempting to determine the nature and origin of the gut contents of *Hexostoma extensicauda*, but concluded that this trematode fed upon mucus extruded on to the gill surface of the host.

According to Sproston (1945) *Kuhnia scombri* fed usually on gill epithelium and mucus when young, but later became a blood feeder. The basis for Sproston's observation was that there was an increase, with the age of the parasite, of pigment granules laid down in the walls of the intestinal rami, the pigment being assumed to have been derived from the haemoglobin of the host. Sproston (1945, 1946) observed that pigment deposits on the walls of the intestine were highly characteristic of all the Diclidophoroidea that she had examined; they were especially noticeable in worms which had been feeding on blood from the gills, i.e. when the crura were bright red in the fresh state. Sproston concluded that the pigment consisted of the breakdown products of haemoglobin, but stated that her attempts to confirm this by applying Brown's test for haematin to preserved material were unsuccessful.

The presence of a dark brown to black gut pigment had been recorded previously in members of nearly all families of the Polyopisthocotylea. Its nature was discussed in some detail by Goto (1895, pp. 61–3), who, however, was unable to decide whether the pigment consisted of food particles, zymogenic granules, or the indigestible remnants of food. Gallien (1935) described various larval stages and adults of *Polystoma integerrimum* as possessing intestinal epithelial cells which secreted a brown pigment, and observed that this pigment appeared only after the parasite had ingested blood. It is important to note, however, that Gallien believed that the ingestion of plasma only (i.e. without erythrocytes) was sufficient to stimulate the secretion of pigment. Gallien stated that this pigment was discharged into the lumen of the intestine or else the pigment cells themselves broke free and were defaecated through the mouth.

Dawes (1940) noted a conspicuous association between the distribution of the pigment cells and of the vitellaria in *Hexostoma extensicauda*, but made no comments on the nature of the pigment granules found inside the cells. He also recorded the presence of clusters of microscopic granules in the detritus in the gut caeca; these granules bore a superficial resemblance to blood cells, but Dawes stated that he was forced to conclude that they were derived from some other source, presumably from mucus extruded from the surface of the host gills.

The difficulties in determining the nature of the food of these trematodes appear then to be that, apart from the single observation by Gallien referred to above, no one has identified a red blood corpuscle of the host in the gut of a parasite, and that tests for decomposition products of blood in the gut contents have been negative.

II. MATERIAL

In the present investigation the Polyopisthocotylea included in Table 1 have been studied, *Polystoma* being obtained from frogs in the Birmingham area, *Discocotyle* from trout from Shropshire and Carmarthenshire, and the remainder from the gills of fishes at Plymouth. Examples of the Avielloidea (Lake Baikal), Chimaericolidae from *Chimaera*, and Hexostomatidae from *Thunnus* were not obtainable, but those species investigated are probably representative both taxonomically and ecologically in that they are taken from six of the nine families of the Polyopisthocotylea and include ectoparasites from marine and freshwater fishes, and an endoparasite from an amphibian.

Table 1. *List of Polyopisthocotylea studied*

Superfamily	Polystomatoidea		Host
Family	Polystomatidae	*Polystoma integerrimum*	*Rana temporaria*
	Hexabothriidae	*Hexabothrium appendiculata*	*Scylliorhinus canicula*
Superfamily	Diclidophoroidea		
Family	Mazocraeidae	*Kuhnia scombri*	*Scomber scombrus*
	Discocotylidae	*Discocotyle sagittata*	*Salmo trutta*
		Anthocotyle merluccii	*Merluccius merluccius*
	Microcotylidae	*Axine belones*	*Belone belone*
	Diclidophoridae	*Diclidophora merlangi*	*Gadus merlangus*
		D. luscae	*G. luscus*

Specimens were examined both in the fresh condition and after fixation in a variety of fixatives, usually in ethyl alcohol for histochemical tests and in Gilson's fixative for histological investigation. In addition, specimens fixed in formaldehyde, acetic acid, osmium tetroxide, picric acid, chromic acid, and potassium dichromate have also been examined. Some investigations were made upon whole animals, and others upon smears of evacuated gut contents and upon serial paraffin sections.

III. MORPHOLOGY AND HISTOLOGY OF THE ALIMENTARY CANAL AND ITS CONTENTS

While there is some variation in the morphology of the feeding mechanisms of the polyopisthocotyleans studied, the morphology and histology of the remainder of the alimentary canal was found to be fairly constant. A pharynx leads, with or without the interpolation of an oesophagus, to two branched intestinal caeca (Pl. XV, fig. 1), the walls of which do not have a continuous cellular epithelium, but instead consist of interwoven fibrils interspersed with 'pigment cells' (Pl. XV, fig. 2). In these features the Polyopisthocotylea differ from the Monopisthocotylea, where the gut resembles that of digenetic trematodes in that the intestinal limbs are usually unbranched and the intestine is lined by a continuous epithelium.

The pigment cells vary in diameter between about 5 and 25μ. When lying free in fresh smears of evacuated gut contents they are seen to be perfectly spherical, but when attached to the intestinal wall, as seen in histological sections, the larger ones are often cup-shaped (Pl. XV, fig. 3), but may be isodiametric or irregular

(Pl. XV, fig. 2), or, infrequently, amoeboid (Pl. XV, fig. 4). A prominent nucleus with nucleolus is nearly always visible in the smaller cells, but is usually obscured by dense masses of pigment granules in the larger cells.

The gut contents in most of the sections examined consisted usually of a very finely granular and faintly acidophilic substance (Pl. XV, fig. 4, *f.i.c.*) that contained also coarser granules of a dark brown or black pigment (Fig. 4, *f.p.g.*). and occasionally vitelline cells and isolated nuclei. In smears of evacuated gut contents there were present some spherical cells containing pigment granules, but such free cells were observed only very rarely in sections.

Pigment granules lying free in the gut contents, or in cells attached to the intestinal wall, or in cells which are themselves lying free in the lumen of the gut, are all of the same appearance: they are dark brown to black and very roughly spherical. Individual granules, measured by projection methods using the ordinary light microscope, were found to have diameters of about 0·5–0·8μ, but it is possible that still smaller granules, which could not be resolved, were present. The granules in living cells and also those immersed freely in gut contents and in various reagents were seen to be in a state of constant agitation, presumably due to Brownian movement.

IV. THE NATURE OF THE GUT CONTENTS

When examined immediately after collection from host fishes captured some 3–6 hr. previously, the gut of most of the parasites was seen to contain a dark brown to black pigment (Pl. XV, fig. 1), but in some specimens of *Axine belones* and *Diclidophora luscae* examined within 1–2 hr. of the capture of their hosts, a bright red fluid was present in the gut. It seemed obvious that the red material was blood taken from the gills of the host, but repeated attempts to observe its absorption spectra with a Hartridge reversion spectroscope fitted to a microscope were unsuccessful. However, on treatment of a whole animal with two or three drops of freshly prepared and warmed Takayama's solution (Hawk, 1938, p. 402), the red pigment underwent, in the course of 2 or 3 min., various colour changes, finally becoming a bright pinkish red. At this stage the preparations were examined with the Hartridge spectroscope, in conjunction with an oil-immersion objective, in order to have the field filled completely with the red colouring matter, and a very well-defined absorption band at 558 mμ, the characteristic band of pyridine haemochromogen, was observed. After a further 3–5 min., characteristic crystals of pyridine haemochromogen (Thorpe, 1938, fig. 20) appeared.

After appropriate treatment, pyridine haemochromogens may be produced from the free intracellular haematin present in all cells of aerobic organisms (Keilin, 1929, p. 232). It became necessary, therefore, to determine whether the haemochromogen produced in the parasites as describe above came from the gut contents or from adjacent tissues of the parasite. This was done by applying Takayama's solution to a region of a parasite observed to be free from 'gut pigment cells' (described below) and vitellaria, and filled with red contents, e.g. the oesophagus of *Axine belones*. The haemochromogen absorption band was given only by the contents of the oesophagus; any haemochromogens produced by the adjacent

tissues of the parasite were not detected with the apparatus used. It was concluded, therefore, that the red contents of the gut of *Axine* were in fact derived from the blood of the host.

By applying similar methods, it was found that the dark pigment present in each of the trematodes listed in Table 1 gave rise to a haemochromogen detectable by spectroscopic observation and by crystal formation. Tests were applied both to fresh specimens and to specimens fixed in alcohol and in Gilson's fixative, including one specimen of *Kuhnia scombri* fixed and preserved in absolute alcohol for 14 months, with similar results in all cases. Upon application of Takayama's solution, the characteristic colour changes took place mainly at the sites of pigment concentration, and accumulations of pyridine haemochromogen crystals were formed almost exclusively in the same areas, i.e. lining the gut caeca as shown in Pl. XV, fig. 5, though some slight crystallization took place where the fluid was in contact with air at the edges of the cover-slip. The inference was that the dark pigment was also derived from the haemoglobin of the host, and, the pigment usually being present in such large quantities, it was concluded that blood formed the major part of the diet of these polyopisthocotyleans.

The gut contents of the polyopisthocotyleans under investigation were examined for the presence of red blood corpuscles, both by inspecting smears of evacuated gut contents and by means of serial paraffin sections of the parasites, but not a single intact erythrocyte was observed. However, in a haematoxylin-eosin stained section of *Discocotyle sagittata* on the gills of *Salmo trutta*, very kindly presented to me by Mr D. Smith, the gut lumen contained what appeared almost certainly to be partially digested red blood cells (Pl. XV, fig. 6). The eosinophilic matrix and basiphilic nuclei exhibited exactly the same staining reactions as the corresponding regions of intact erythrocytes in blood vessels of the host in the same section; the sizes of the nuclei (about 3μ in diameter) correspond, and are significantly different from those of the nuclei in other tissues present in the same series of sections. The corresponding eosinophilic matrix in sections of specimens of several species of trematodes prepared *in situ* on the gills of their hosts gave a negative result to the periodic acid-leucofuchsin test for polysaccharides (Gomori 1952), whereas mucus gland cells of the gills of the hosts gave strongly positive results, indicating that this matrix was probably not derived from mucus.

In the present study the pigment masses in the gut of the eight species of Polyopisthocotylea investigated have already been shown, by spectroscopic and crystallization tests conducted on whole mounts, to contain a derivative of haemoglobin. However, while it was possible to establish that the haemochromogen detected in the gut of whole mounts by Takayama's tests came from the free (extracellular) pigment in the absence of intracellular pigment (see above), it was not possible to conclude that the intracellular pigment also contained a haemochromogen precursor because, although pigment cells could be identified readily in whole mounts, it was not possible to be certain that extracellular pigment was not present also. Takayama's pyridine haemochromogen tests were then applied to paraffin sections of the parasites in which the two sites of pigment deposits could be distinguished from each other. It was found, however, that even in sections of 25μ thickness, no

absorption bands or crystals could be detected, presumably on account of the insufficiency of pigment present, and so other tests were applied as follows.

Pigment granules in both intra- and extracellular sites were removed in less than 1 hr. from paraffin sections of 10μ thickness by immersion either in a saturated alcoholic solution of picric acid or in a N/2 ethanolic solution of potassium hydroxide. Pigment granules in both sites were also bleached within 2 hr. by a 3% (w/v) solution of hydrogen peroxide. These properties of solubility and capacity to be bleached are characteristic of haematin (Gomori, 1952).

Both intra- and extracellular pigment granules gave positive prussian blue reactions for ferric iron, but only following treatment to unmask bound iron. Attempts to unmask the iron by treatment with various concentrations of sulphuric acid–alcohol and hydrochloric acid–alcohol mixtures at a variety of temperatures and for periods up to 4 days were all unsuccessful. However, pre-treatment of the sections for 30 min. with a 30% (w/v) solution of hydrogen peroxide brought to a pH of about 7·5 with sodium carbonate, as described by Gomori (1952), produced strongly positive prussian blue reactions at the sites of pigment granules. This bound iron content is a property of haematin, the iron of haemosiderin occurring in a form which reacts with potassium ferrocyanide without recourse to unmasking procedures, and melanin containing no iron (Gomori, 1952).

In sections subjected to the argentaffin test for reducing substances (Lison, 1936) both intra- and extracellular pigment granules were negative, becoming a lighter brown, whereas vitelline droplets in the same sections were intensely positive. Haematin is characterized by being negatively argentaffin, and melanin by being positively so (Gomori, 1952).

A possible peroxidase property of the pigment(s) was investigated by means of Lison's zinc-leuco acid fuchsin method (Gomori, 1952) and by means of benzidine and *o*-tolidin reactions (Hawk, 1938), again with no difference in response between intra- and extracellular granules. The zinc-leuco method, which gives best results with fresh haemoglobin (Gomori, 1952) was negative, but the benzidine method, which stains 'even somewhat stale, degraded haemoglobin' (Gomori, 1952), and the *o*-tolidin method, both gave usually, but not invariably, positive results. In the Polyopisthocotylea investigated, the variations in response to peroxidase tests seemed to be associated neither with different sites of the pigment, nor with the age of the various samples. All fresh material used gave positive benzidine reactions, but whereas sections of a Gilson-fixed specimen of *Diclidophora merlangi* preserved in 70% alcohol for 3 weeks gave negative results, some ordinary stained histological sections of the same species, similarly fixed and mounted in Canada Balsam for 12 months, gave positive results. In those samples of pigment which gave a positive peroxidase reaction, the reaction was not inhibited by exposing the material to a steam jet for 10 min.

It is perhaps worth recording that some difficulty was experienced in determining the precise sites of the peroxidase reactions. It was found that the blue oxidation product of benzidine and hydrogen peroxide is a water-soluble dye that is taken up very readily by basophilic bodies. In smears of stickleback blood subjected to a benzidine and hydrogen peroxide mixture, an over-all blue colour was

soon evident macroscopically, but on microscopic examination it was found to be the nuclei and not the cytoplasm of the erythrocytes that were stained blue. Similarly, when mammalian blood smears were treated with the same mixture, a general blue colour was produced, but it was not concentrated in the non-nucleated erythrocytes. It was concluded therefore that the sites of blue staining following benzidine and hydrogen peroxide treatment were not necessarily the locations of peroxidases.

In order to identify the precise sites of peroxidase activity, the following method was adopted. The rate of diffusion of the blue dye resulting from the benzidine and hydrogen peroxide reaction was decreased by mixing the reagents, before application, with melted glycerine jelly. By this method the blue dye could be observed to form and to accumulate in association with pigment granules whether the latter were within epithelial cells or free in the gut lumen.

It has been shown therefore that both the intra- and extracellular pigment granules, having the same physical appearance, are also similar in possessing the following properties: they are dissolved by alcoholic picric acid and by alkaline alcohol, and are bleached by hydrogen peroxide; they both contain masked iron, are negatively argentaffin, and usually give a positive peroxidase reaction. These intra- and extracellular granules are therefore regarded as being identical, and a consideration of their physical and chemical properties, taken in conjunction with the results of spectroscopic and crystallization tests conducted upon haemochromogens derived from (mixtures of?) intra- and extracellular pigment, indicates that the gut pigment of the polyopisthocotyleans investigated consists of haematin.

V. COMPARISON WITH THE MONOPISTHOCOTYLEA

For comparative purposes, observations were also made on some monogeneans in which pigment is apparently lacking, the monopisthocotyleans *Leptocotyle minor* from the skin of *Scylliorhinus canicula* and *S. catula*, and *Acanthocotyle* sp. from the skin of *Raja clavata*. Whole mounts of some forty specimens of each of these trematodes were examined, but none appeared to contain any pigment. Six specimens of each trematode, some fresh and some fixed in alcohol, were immersed in Takayama's solution, but no haemochromogen absorption band was detected, and no pyridine haemochromogen crystals were formed. Whole mount preparations of ten specimens of *Calicotyle kroyeri* from the cloaca of various species of *Raja*, and a single museum specimen of *Tristoma papillosum* from a swordfish, were also examined for pigment, but none was observed. It seems probable therefore that these, and possibly other skin and cloaca-inhabiting monopisthocotyleans as well, are not blood feeders. They may feed on eroded tissue or mucus or, for all the available evidence, be merely commensals.

VI. DISCUSSION

On the evidence of (1) the presence, in specimens of all the eight species of Polyopisthocotylea investigated, of abundant haematin in the gut, (2) the presence, in the gut of specimens of two species, of a red fluid that on appropriate treatment yielded haemochromogen, and (3) the presence, in the gut of one specimen, of

recently digested erythrocytes, it appears probable that all these parasites feed mainly, if not wholly, upon the blood of their hosts.

The failure to observe more frequently intact erythrocytes of the host in the gut contents of the parasites may well have been due to haemolysis having taken place in the interval between the time of capture of the host (= cessation of feeding of the parasite?) and the time of examination of fresh smears of gut contents or of fixation before histological sectioning. In the present investigation this interval, with one exception, was never less than 1 hr. The exceptional case was that of a trout captured in Fifeshire by Mr D. Smith and fixed within a few minutes of the death of the fish. This trout harboured on its gills a specimen of *Discocotyle sagittata* that was found, on sectioning, to contain haemolysed blood in its gut (Pl. XV, fig. 6).

The haemolysis would appear to be followed by phagocytosis on the part of the cells of the discontinuous intestinal epithelia. Relatively very few typically amoeboid epithelial cells have been observed in histological sections of any of various Polyopisthocotylea studied, but the infrequency of the amoeboid condition could be due to the process of fixation. According to Willier, Hyman & Rifenburgh (1925), phagocytosis and intracellular digestion are characteristic of the Turbellaria.

It appears probable that the globin fraction of the haemoglobin forms the chief nutriment of the trematodes investigated, since prussian blue tests have failed to reveal any concentrations of ferric iron outside the intestinal epithelial cells. The haematin fraction is eventually eliminated, either by its egestion from the epithelial cells and its subsequent evacuation through the mouth, or by the defaecation of sloughed-off intact epithelial cells laden with haematin granules. Both these methods of eliminating haematin have been observed frequently in living specimens kept in (sea) water. A comparable nutritional process involving the non-utilization of at least the greater part if not all of the haematin has been described in other haematophagous forms such as *Fasciola hepatica* by Stephenson (1947), various Arthropoda by Wigglesworth (1943), and in malarial parasites by Hoare (1949, p. 222).

In view of the above demonstration that the gut pigment of eight species of Polyopisthocotylea consists of haematin derived from the blood of their hosts, Gallien's (1935) supposition that, in larvae of neotenics of *Polystoma integerrimum*, the pigment of the gut epithelial cells is secreted after absorption of only the plasma of the blood of their hosts is almost certainly incorrect, since the synthesis of haematin from plasma seems extremely improbable.

Again, the occurrence in *Hexostoma extensicauda* of pigment cells in conspicuous association with the vitellaria (which are themselves closely associated with the intestine) may indicate that this gill trematode feeds at least in part upon blood and not exclusively upon mucus as was believed by Dawes (1940).

Concurrently with the above study of the nature of the food of some of the Polyopisthocotylea, some investigations have been made upon the process of egg capsule formation in the same animals. It is not proposed to anticipate here the results of this work, but it is perhaps relevant to state that *Diclidophora merlangi* has been observed to have a high rate of capsule production (over 25 in a 9 hr.

period) and that the vitelline droplets which contribute to the formation of these capsules have been found to contain an abundance of an orthodihydroxyphenol. The maintenance of such an economy would appear to be more consistent with blood feeding than with a diet of mucus.

The present investigation does not support the general application to the other Polyopisthocotylea of Sproston's (1945) observation that in *Kuhnia scombri* there is an increase, with the age of the parasite, of pigment deposition. In fact some relatively large, sexually mature, and presumably fully grown specimens of *Diclidophora merlangi* have been observed to be quite without pigment. This absence of pigment could be attributed to its having been defaecated.

Finally, it is suggested that in any future study of the gut pigment in fixed specimens of the Polyopisthocotylea, cognizance be taken of the effects of histological fixatives upon blood and haematin; haematin could be removed by fixatives that include alcoholic picric acid, and pigmentation could be increased by fixatives that include formaldehyde which might form 'formalin pigment' (Gomori, 1952).

VII. SUMMARY

1. It has been shown by spectroscopic and histochemical methods that eight representative species of Polyopisthocotylea feed mainly on the blood of their hosts.

2. The blood is probably haemolysed fairly rapidly and subsequently absorbed by amoeboid ingestion, the globin moiety of the haemoglobin forming the chief nutriment of the parasite and the unaltered haematin being eliminated either by its discharge from epithelial cells into the gut lumen or by the sloughing off of intact epithelial cells.

3. In a more limited sample of skin-and cloaca-inhabiting Monopisthocotylea there was no evidence of a blood-feeding habit.

The writer is indebted to the Director and Staff of the Marine Station Plymouth for collecting and laboratory facilities, and to Dr H. Rees for the supply of trout. The work was aided by a grant from the Browne Research Fund of the Royal Society.

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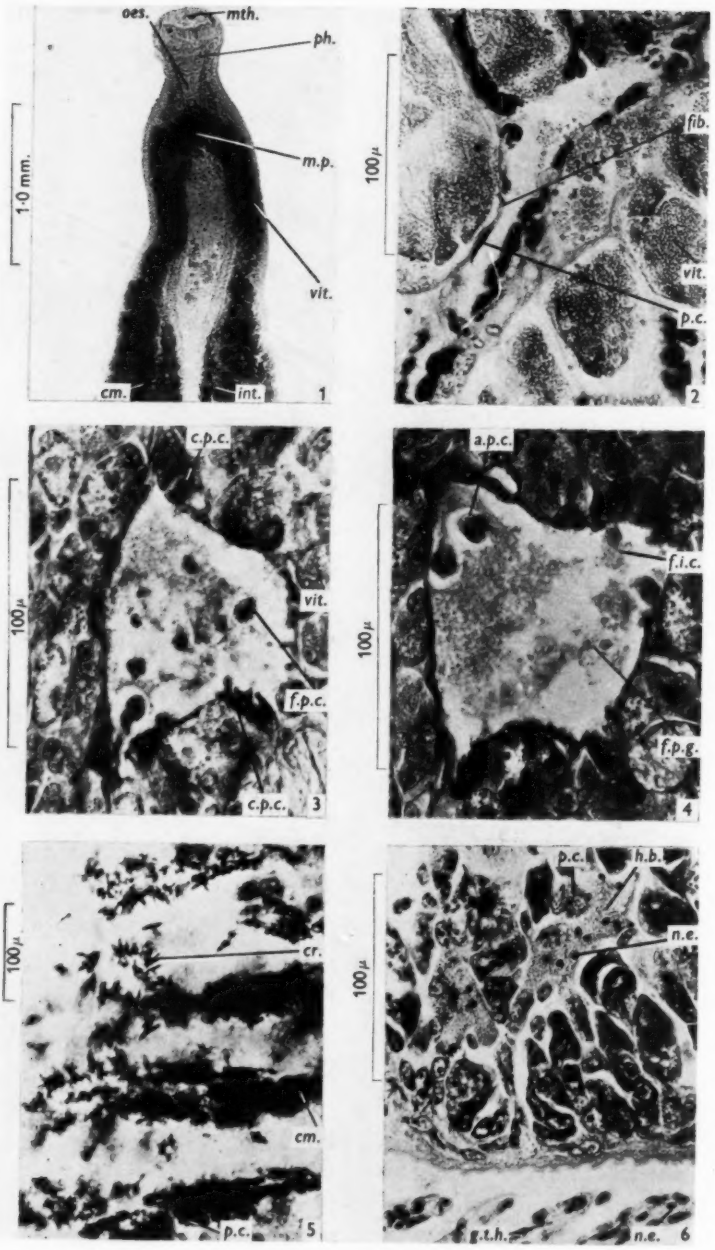
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EXPLANATION OF PLATE XV

(*Abbreviations*: *a.p.c.* amoeboid pigment cell; *cm.* intestinal caecum; *c.p.c.* cup-shaped pigment cell; *cr.* crystals of pyridine haemochromogen; *fib.* fibrillar wall of intestine; *f.i.c.* finely granular intestinal contents; *f.p.c.* pigment cell free in intestine; *f.p.g.* pigment granules free in intestine; *g.t.h.* gill tissue of host; *h.b.* haemolysed blood; *int.* intestine; *m.p.* mass of pigment; *mth.* mouth; *n.e.* nucleus of erythrocyte; *oes.* oesophagus; *p.c.* pigment cell; *ph.* pharynx; *vit.* vitellarium.)

Fig. 1. Anterior end of *Diclidophora denticulata*.

Fig. 2. Transverse section of intestinal region of *Diclidophora merlangi* to show fibrillar intestinal border with scattered pigment-filled epithelial cells.

Fig. 3. Transverse section of intestinal region of *Anthocotyle merluccii* to show cup-shaped epithelial cells, pigment cells free in lumen of intestine, and pigment granules free in the intestine.

Fig. 4. Transverse section of intestinal region of *A. merluccii* to show an amoeboid pigment cell and pigment granules free in the intestine.

Fig. 5. Part of whole mount of *A. merluccii* after treatment with Takayama's reagent, to show crystals of pyridine haemochromogen in gut caeca.

Fig. 6. Longitudinal section of *Discocotyle sagittata* on gill of *Salmo trutta* to show partially digested erythrocytes of host in intestine of parasite. (From a slide presented by Mr D. Smith.)

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THE EFFECT OF CORTISONE ON *PLASMODIUM BERGHEI* INFECTIONS

By O. JOY ROBERTS

The Ross Institute of Tropical Hygiene, London School of Hygiene and Tropical Medicine

(With 6 Figures in the Text)

INTRODUCTION

It has been generally thought that cortisone, by inhibiting the cellular response to infection, permits in this way unrestrained multiplication of the infecting agent, with the appearance of much more severe symptoms. In infections of mice with *Plasmodium berghei*, Findlay & Howard (1952) found that cortisone (as cortisone acetate) when injected into infected mice, caused a rapid increase in the numbers of parasites present in the peripheral blood. The cortisone was given in three doses, each of 5 mg., 30 min. before inoculation with infected blood, 6 hr. later and 4 days later. Death occurred in the experimental mice in about 5 days, whereas the control mice, which had received similar doses of saline, died in 9–10 days.

In a previous paper (Roberts, 1954) it was reported that it had not been possible to confirm the results of Findlay & Howard, using the same doses of cortisone acetate and the same timing, in mice infected with *P. berghei*. There was little difference between the cortisone-treated mice and the controls, either in the numbers of parasitized red blood cells present in the peripheral blood, or in the total parasites present, the multiple infections of many cells having been counted.

Cortisone did not appear to exert an inhibitory influence on the multiplication of the parasite in the peripheral blood. The numbers of schizonts present in the blood, and the number of merozoites in each, did not differ to any marked degree in the experimental and control animals. The results of similar experiments carried out on hamsters were also reported and appeared to show a depressive action of cortisone on the multiplication of the parasite at an early stage in the infection. Control animals showed many more parasites in the peripheral blood than did the experimental animals, and blood films from the controls also showed more schizonts present with larger numbers of merozoites in each. At a later stage in the infection both control and experimental animals often showed a very high degree of anaemia and parasitaemia.

In the light of these results it was suggested that some humoral agent might be present, restricting the multiplication of the parasite in the peripheral blood, despite the presumed inhibition of the cellular response, by cortisone.

In an attempt to demonstrate the presence of some humoral agent in the blood, which would have the effect of restricting parasite multiplication, various sera were prepared from cortisone-treated mice and others and were injected into

infected mice which had not been given cortisone. The courses of the infections in the recipient mice were studied before and after the doses of prepared serum, and were compared with those in control mice.

TECHNIQUE

The strain of *P. berghei* ('*C*') used in this work was virulent with numerous gametocytes. It was obtained from the Belgian Congo by courtesy of Dr Vincke and had been kept for 6 months in the laboratory, being passaged in young hamsters.

The cortisone used was kindly given by the Medical Research Council.

Mice weighing about 20 g. were infected with *P. berghei* by intraperitoneal inoculation with citrated hamster blood. The same route was used for injecting cortisone acetate. When the mice sera were prepared, blood was removed from the heart into a sterile syringe, and centrifuged for 10 min. The serum was pipetted off, again in sterile conditions, and was used within 1 hr. of its preparation, or in a few cases was placed in a refrigerator overnight. When the course of infection in mice was followed throughout, blood films were taken from the tails, stained with Giemsa and examined once or twice daily. Parasitized red cells were counted and expressed as numbers per microscope field (10 eye-piece).

Table 1

Days after infection	Parasitized cells per field	
	Control	Experimental
1	0·28	0·22
2	2·3	2·5
3	—	—
4	—	11·4
5, a.m.*	11·1	11·5
5, p.m.	11·2	10·0
6	10·0	6·8
7	11·8	4·6
8	17·1	9·1
9	18·0	13·1

* Serum given.

In the first set of experiments, serum was prepared from mice which had been infected with *P. berghei* and which had received 15 mg. of cortisone acetate intraperitoneally. This had been given in three doses, 30 min. before inoculation with infected blood, 6 hr. after and 2 days later. On the fourth day of the infection, 2 days after the last dose of cortisone, the serum was prepared and was injected in doses of 0·1 ml. into mice which had been infected with the same strain of *P. berghei* 5 days previously. Thin blood films taken from the experimental mice and the controls 6 hr. after the former had been given serum, showed a noticeable decrease in the number of parasites in the peripheral blood of the experimental animals, which persisted in all for some days afterwards. This is shown in Table 1 and Fig. 1. This decrease in the parasitaemia appeared the more remarkable when the very small amount of serum involved was considered. It is well known that in

P. berghei infections there is commonly a 'crisis' stage, which in this strain often appears between the 5th and 6th days. This 'crisis' is shown by the graph of the control mice, in Fig. 1, and for this reason and in view of the small amount of serum involved, the results were considered to be unreliable.

The experiments were therefore repeated, the serum being prepared as before, but injected in doses of 0·15 ml. into animals infected only 4 days previously instead of 5 as in the previous cases. In this way it was hoped to differentiate between normally occurring decreases in parasitaemia and any induced by serum

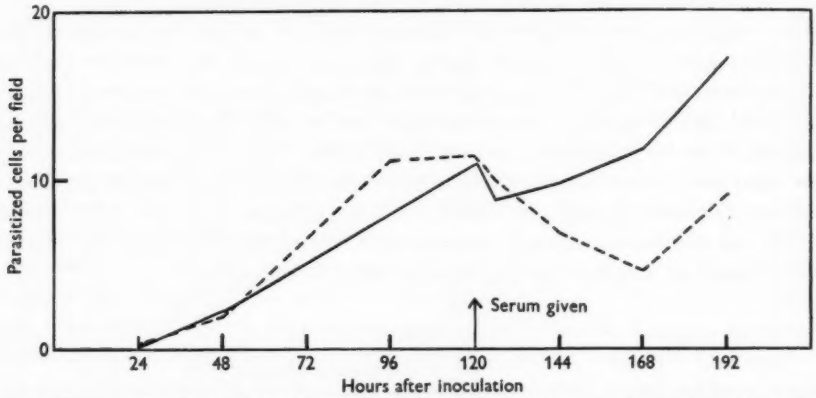

Fig. 1. Course of a *P. berghei* infection mice given serum from cortisone-treated, infected mice. All points are averages of five mice. ——, control mice; - - - - -, experimental mice.

injections. Parasite counts showed an immediate drop in the numbers of parasites present in the peripheral blood of the experimental animal, but the parasitaemia continued to increase in the control mice until 24 hr. later, when a 'crisis' stage occurred. See Table 2 and Fig. 2.

Table 2

Days after infection	Parasitized cells per field	
	Control	Experimental
1	0·4	0·4
2	3·7	3·7
3	11·3	12·2
4, a.m.*	18·8	19·8
4, p.m.	21·0	16·6
5	16·2	17·6
6	13·0	15·3

* Serum given.

RESULTS OBTAINED BY INJECTING SERUM PREPARED SIX DAYS AFTER CESSATION OF CORTISONE TREATMENT

A further set of experiments was performed with serum prepared from infected mice 6 days after the last dose of cortisone. Cortisone had been given in the same quantities and with the same timing as in the previous experiments.

Serum was injected in 0·2 ml. doses into mice 4 days after inoculation with

infected blood. The experimental mice showed a very marked decrease in parasitaemia compared with the controls. See Table 3 and Fig. 3.

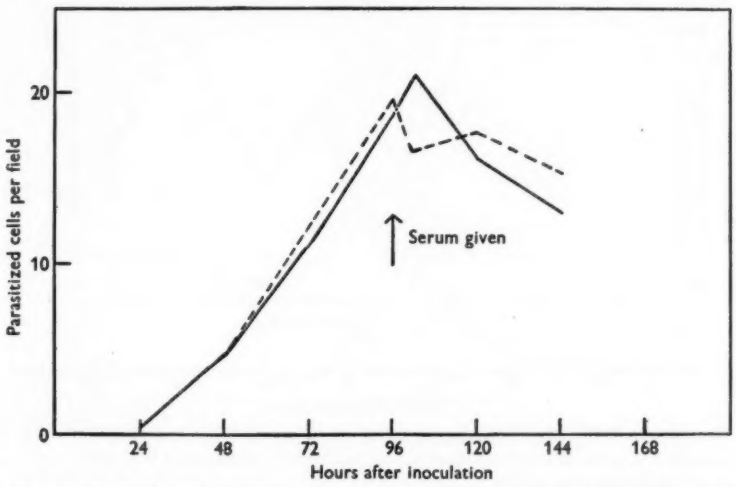

Fig. 2. Course of a *P. berghei* infection in mice given serum from cortisone-treated, infected mice on the 4th day of the experimental infection. All points are averages of five mice. ——, control mice; - - - - -, experimental mice.

Table 3

Days after infection	Parasitized cells per field	
	Control	Experimental
2	3·3	3·2
3	21·2	15·7
4, a.m.*	44·3	38·1
4, p.m.	64·2	33·1
5	45·6	20·6
6 (3 dead)	81·0	26·8
7	42·0	27·8
8	48·1	32·9
9	—	(3 dead)

* Serum given.

RESULTS OBTAINED FROM THE INJECTION OF SERUM CLEAN, UNINFECTED, UNTREATED MICE

It was felt necessary at this stage to investigate the effect produced by the injection of serum from clean uninfected mice, which had received no cortisone, in order to eliminate the possibility of the observed drop in parasitaemia being due to the introduction of foreign serum.

'Clean' serum was given to experimental mice, on the fourth day of their infection, and the results are shown in Table 4 and Fig. 4.

There was no noticeable difference between the experimental and the control mice; the parasite counts continued to rise in both sets of animals. See Table 4 and Fig. 4.

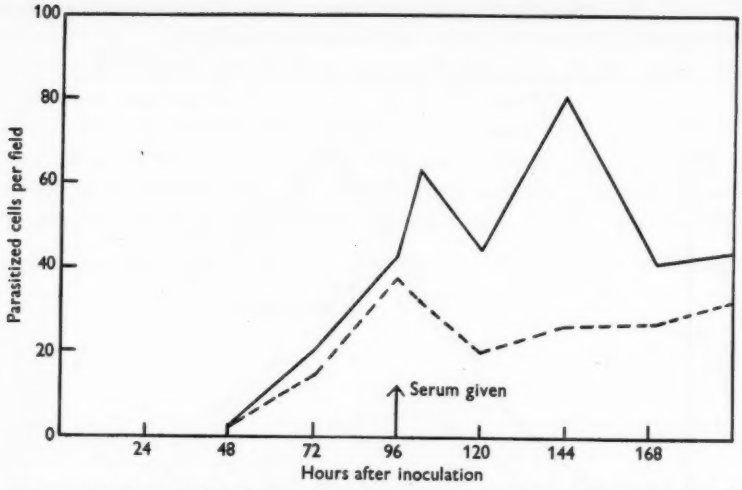

Fig. 3. Course of a *P. berghei* infection in mice given serum from cortisone-treated, infected mice 6 days after the last dose of cortisone. All points are averages of five mice. ——, control mice; - - - - -, experimental mice.

Table 4

Days after infection	Parasitized cells per field	
	Control	Experimental
1	0·6	0·5
2	3·0	3·4
3	16·4	10·6
4, a.m.*	40·1	23·4
4, p.m.	56·0	37·3
5	59·3	47·7
6	50·6	59·2
7	39·9	37·3
8	(4 dead)	23·8 (3 dead)

* Serum given.

RESULTS OBTAINED BY INJECTING SERUM PREPARED FROM CORTISONE-TREATED MICE WITH NO INFECTION

Cortisone acetate was given to mice in doses of 5 mg. daily for 5 days. Three days after the last dose of the drug the serum was prepared and was injected in doses of 0·1 ml. into infected mice which had received an inoculation with parasites 4 days earlier. No decrease in the level of parasitaemia was observed. See Fig. 5.

RESULTS OBTAINED BY INJECTING SERUM PREPARED FROM MALARIA-INFECTED AND UNTREATED MICE

Serum was prepared from mice infected with *P. berghei* on the fourth day of the infection. These mice had received no cortisone nor other drugs. The serum was injected in doses of 0·15 ml. into infected mice which had received parasites 4 days previously.

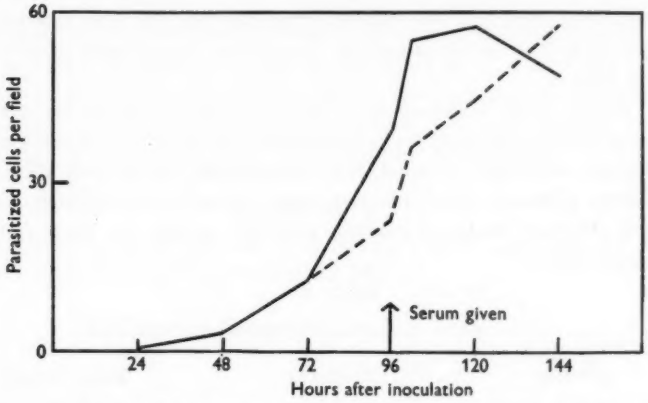

Fig. 4. Course of a *P. berghei* infection in mice given clean serum on the 4th day of the experimental infection. All points are averages of five mice. ——, control mice; - - - - -, experimental mice.

Table 5

Days after infection	Parasitized cells per field	
	Control	Experimental
1	2·6	1·5
2	15·7	11·0
3	33·9	31·5
4, a.m.*	42·0	42·7
4, p.m.	56·0	52·0
5	47·5 (3 dead)	51·7 (2 dead)

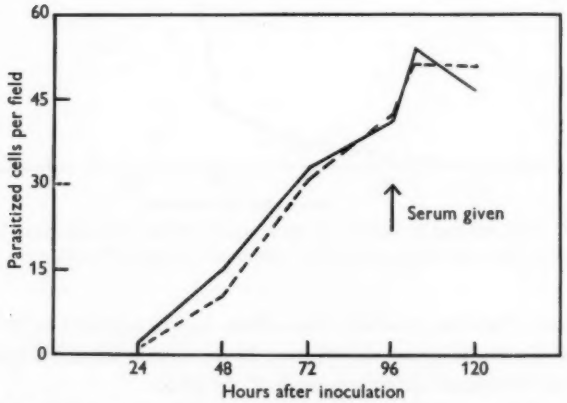

Fig. 5. Course of a *P. berghei* infection in mice given serum from cortisone-treated but uninfected mice. All points are averages of five mice. ——, control mice; - - - - -, experimental mice.

In experimental and control animals the parasite counts showed an uninterrupted rise in all but one case. In the experimental animals the rise was rather slower immediately after the serum injection, but 18 hr. later both counts were very similar. See Fig. 6.

It would appear that injections of extra serum from malaria-infected mice into others in all respects alike may have a transient effect on the parasite count in the peripheral blood, although an injection of clean serum has no such effect. When the serum has been obtained from infected mice treated with cortisone this effect of parasitaemia decrease becomes marked and may persist for some time after the serum has been given.

Table 6

Days after infection	Parasitized cells per field	
	Control	Experimental
1	1·1	1·04
2	2·0	1·6
3	4·7	3·8
4, a.m.*	11·3	10·8
4, p.m.	30·9	12·4
5	43·4	37·1
6	34·4	45·8

* Serum given.

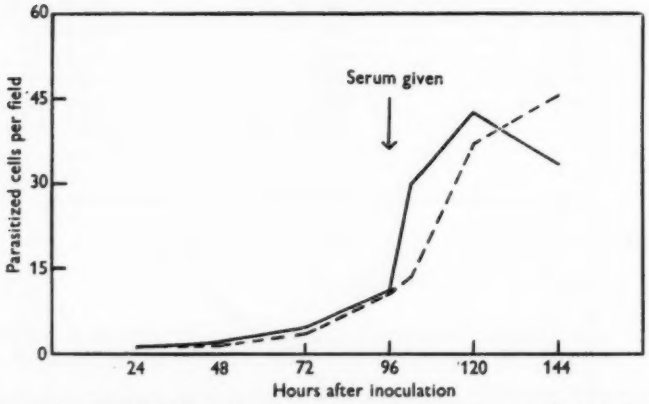

Fig. 6. Course of a *P. berghei* infection in mice given serum from malaria-infected, but untreated mice. All points are averages of five mice. ——, control mice, - - - - -, experimental mice.

Cortisone alone does not produce this effect, but it appears to enhance the production of some humoral agent which may be produced in small quantities during the course of an untreated infection with *P. berghei*.

The very small quantities which must be involved remain most puzzling. In these experiments the doses of sera have varied from 0·1 to 0·2 ml.—never more, yet these quantities have proved capable of producing a marked and long-lasting decrease in parasitaemia.

SUMMARY

Cortisone appears to enhance the production of some humoral agent in the serum of infected mice which, when injected in extremely small quantities into experimental, infected animals, produces a decrease in the parasitaemia in the peripheral blood. This effect is not produced by clean serum, and only slightly, if at all, by the serum from infected mice which have received no cortisone.

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A NEW ANOPLOCEPHALID CESTODE FROM THE GERBIL

By GUTA WERTHEIM, Ph.D.

Department of Parasitology, The Hebrew University, Jerusalem, Israel

(With 3 Figures in the Text)

A new cestode of unusual anatomical characteristics was collected from a group of *Gerbillus pyramidum* caught in August 1953, in the vicinity of Tel Aviv. It appears to be a new species of a new genus for which the name of *Rajotaenia gerbilli* n.g. n.sp. is proposed. Thanks are due to Mr M. Costa for collecting the infected rodents.

Of nine gerbils examined, four were infected in varying degree, ranging between four to fifty-three specimens per host. The worms are located in the duodenum and the upper part of the small intestine. No additional parasites were found in ten gerbils caught 2 months later in the same locality.

METHODS

The worms were removed from the small intestine into tap water and were allowed to relax for from half to one hour. They were then fixed in formalin, 70% alcohol or Bouin's picric-formol. No pressure was applied during fixation and mounting. Whole mounts were prepared from alcohol-fixed specimens stained with dilute Ehrlich's haematoxylin as described by Voge (1952). Serial sections, 8 μ thick, were stained with Ehrlich's haematoxylin or Mallory's aniline-blue. Measurements of the worms were taken from unfixed specimens, relaxed in water, and of internal organs and suckers from mounted specimens.

DESCRIPTION

The worm is short and almost triangular in shape. The serrated border of the posterior proglottid is in the form of an inverted V (Fig. 1). When in active movement, both lateral portions of the last proglottid contract and expand independently somewhat recalling the swimming movements of a ray, whence the proposed name.

The entire worm is 4·5–5·0 mm. long, tapering anteriorly, with the maximum width of 3·6–4·5 mm. at the posterior end of the mature proglottid. The scolex is triangular with its anterior margin rounded. It is not prominent as in most cestodes, but it rests on a concave depression in the anterior margin of the first proglottid. The scolex has no rostellum and it bears four unarmed suckers which are 0·10–0·13 mm. in diameter. The neck is very short and, contrary to the usual pattern, wider than the scolex. It contains a narrow band of deeply staining, densely packed nuclei which represent the rudiment of the genitalia. The entire strobila is built of three to four proglottids. When four proglottids are present, the last one is gravid and detaches easily, while behind the scolex a transverse constriction can be observed, indicating the initial development of a young pro-

glottid. In all proglottids the lateral margins are longer than the midline. The border between the first and second segment is concave while the posterior borders of the following two segments are convex.

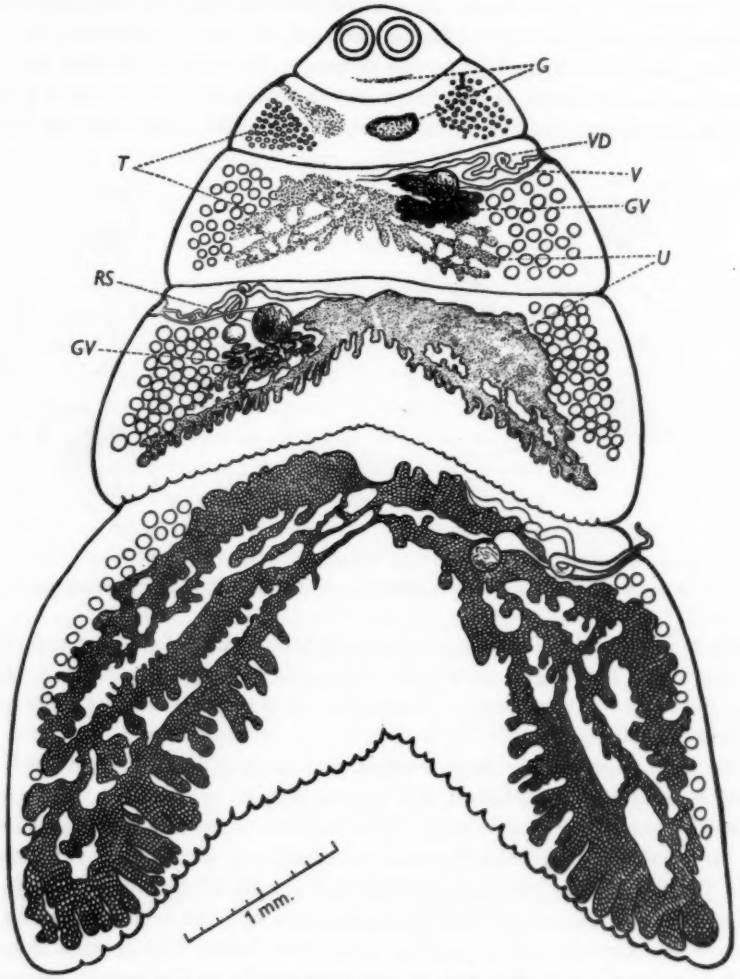

Fig. 1. *Rajotaenia gerbilli*, entire specimen. *G*, genital rudiment; *Gv*, germovitellarium; *Rs*, seminal receptacle; *T*, testes; *U*, uterus; *V*, vagina; *Vd*, vas deferens.

The genital pores alternate regularly. In the first proglottid the rudiment of the female genitalia assumes the shape of an oval field. This proglottid also contains the rudiment of the genital ducts and numerous spherical testes 0·044–0·055 mm. in diameter, concentrated in the two lateral fields of the proglottid.

In the second proglottid the genital apparatus is already well developed. The numerous testes, altogether up to 100 in number, are larger and attain 0·066 mm. in diameter. The central part of the proglottid is occupied by the developing

uterine lobes which in this segment occur as a bundle of narrow tubes arising from a point near the anterior margin of the proglottid. The vas deferens follows the anterior margin of the proglottid, and after making a few loops enters the genital atrium. There is no cirrus sac or any other accessory organ of the male genitalia. The vagina is a straight tube which passes from the genital atrium to the seminal receptacle just posteriorly to the vas deferens. The large spherical seminal receptacle containing sperm, is situated at the aporal end of the vagina at a distance about one-fourth of the segment's width from the margin. Posterior to it is situated

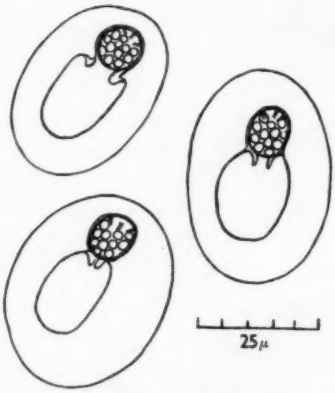

Fig. 2. Eggs taken from fresh specimen.

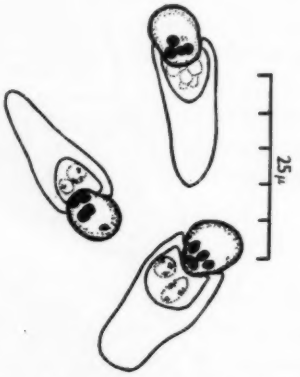

Fig. 3. Eggs in mounted sections.

a compact, irregularly lobed, deeply staining body, built of oocytes and vitellum-producing cells. This organ apparently functions as a germovitellarium (Hyman, 1951). Medially to the seminal receptacle an agglomeration of cells possibly represents the Mehlis gland.

In the next segment the degenerating remnants of the testes, as well as the seminal receptacle, vas deferens and vagina persist, while most of the space is occupied by the developing uterus. Only fragments are left of the germovitellarium. In the fourth proglottid the uterus is completely developed. It is horse-shoe shaped, its lateral lobes joining at the anterior margin of the proglottid. Each lobe consists of three transverse, very irregular sacs interconnected by some of the numerous sac-like expansions arising from their sides. The seminal receptacle, genital ducts and a few remnants of the tests persist in the gravid segment.

No eggs were found in the faeces of the infected animals. Developed eggs were found only in a few seemingly fully developed proglottids. When such proglottids were pressed between two slides either of two types of egg could be observed. One type (Fig. 2) is elongated oval 45 μ long and 36 μ wide; each egg containing a rounded oncosphere 12–14 μ wide and an elongated appendage 25 μ long and 13 μ wide which may be regarded as a homologue of the pyriform apparatus of other anoplocephalids. The oncosphere hooklets are 3 μ long. The second type of egg (Fig. 3) is much more elongate, each egg containing a rounded oncosphere with a triangular appendage. The ova are 29–32 μ long and 10–13 μ wide, measured

from material fixed in alcohol. The oncosphere is 10 μ long and 8 μ wide also in fixed material. In stained sections four large cells are prominent in the oncosphere. The excretory system is represented by twenty narrow, longitudinal, anastomosing vessels distributed over the whole width of the worm.

Host: *Gerbillus pyramidum* Geoffrey, 1822, subsp. *Tarabuli* Thomas, 1902.

Location: Duodenum and upper part of small intestine.

Locality: Holon near Tel Aviv.

Type specimen: Helminthological collection, Department of Parasitology, The Hebrew University, Jerusalem, Israel.

Paratypes: British Museum, London; U.S. National Museum, Washington.

DISCUSSION

The non-rostellate, unarmed scolex and the pyriform apparatus are characters that place this genus in the family Anoplocephalidae. According to the latest proposals of classification (Spasski, 1951), the family Anoplocephalidae should be divided into two subfamilies: Anoplocephalinae Blanchard, 1891 and Monieziinae Spasski, 1951, differing in the shape of the developing uterus. In the Anoplocephalinae, this organ appears as a single transverse tube, later giving off lateral branches, while in Monieziinae it appears as a network, the loopholes of which may eventually be closed when the uterus fills with eggs. In *Rajotaenia* the uterus appears as several branches radiating from the centre of the proglottid and later as a network in which three main transverse tubes with numerous outpocketings predominate. Development of this sort is not characteristic of either of the two subfamilies mentioned, though it may possibly prove to be an aberration of the Monieziinae pattern. A conclusive opinion on the systematic position of *Rajotaenia* is withheld until additional forms may be described.

The author wishes to express her thanks to Professor G. Witenberg for his constant help and kind advice.

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THE ACTION OF 2:4-DIAMINO-6:7-DI*ISO*PROPYLPTERIDINE UPON *PLASMODIUM GALLINACEUM* AND ITS RELATION TO OTHER COMPOUNDS WHICH ARE PTEROYLGLUTAMIC ACID ANTAGONISTS

By ANN BISHOP*

Molteno Institute, University of Cambridge

(With 1 Figure in the Text)

PAGE

I. Introduction 450
II. Methods 452
III. The sensitivity of the 0/129-resistant strains to other antimalarial compounds 453
IV. The sensitivity of an 0/63-resistant strain to other antimalarial compounds . 456
V. A comparison of the effect of *p*-aminobenzoic acid (*p*-A.B.) and pteroylglutamic acid (P.G.A.) upon sulphadiazine, proguanil and 2:4-diamino-6:7-di*iso*propylpteridine (0/129) 456
VI. Discussion 459
VII. Summary 461
References 462

I. INTRODUCTION

The discovery by Woods (1940), that the antibacterial action of sulphanilamide is antagonized by *p*-aminobenzoic acid—a discovery confirmed by Selbie (1940), Rubbo & Gillespie (1940), Blanchard (1941) and others—and his hypothesis that *p*-A.B. is an essential metabolite for bacterial growth, stimulated research upon the relationship between drug action and the metabolism of micro-organisms. It was found that certain bacteria are able to synthesize folic acid when grown in the presence of high concentrations of *p*-aminobenzoic acid (Mills, Briggs, Luckey & Elvehjem, 1944; Sarett, 1947), and that in some bacteria synthetic folic acid (pteroylglutamic acid) and related compounds are sulphonamide antagonists (Lampden & Jones, 1946*b*). The elucidation of the structure of folic acid by Angier and his co-workers (1946) showed that *p*-aminobenzoic acid is a component of the folic acid molecule.

There is evidence that the mode of action of proguanil† upon plasmodia is related to folic acid metabolism, but the relationship cannot be a direct one. Thus, according to Greenberg (1949*b*), the inhibitory action of sulphadiazine upon *Plasmodium gallinaceum* is completely antagonized by P.G.A.,† whereas that of

* Member of the Scientific Staff of the Medical Research Council.

† The chemical formulae of the compounds used in these experiments and the contractions or symbols used in their stead are as follows:

(*p*-A.B.): *p*-aminobenzoic acid.
(P.G.A.): pteroylglutamic acid, synthetic folic acid, *N*-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid.
(0/129): 2:4-diamino-6:7-di*iso*propylpteridine.
(0/63): 2:4-diamino-6:7-diphenylpteridine.
Sulphadiazine: 2-(*p*-aminobenzenesulphonamido)-pyrimidine.
Proguanil: (paludrine, chlorguanide), N^1-*p*-chlorophenyl-N^5-*iso*propylbiguanide.
Pyrimethamine: (Daraprim), 5-(*p*-chlorophenyl)-2:4-diamino-6-ethyl-pyrimidine.
(48–210): 2:4-diamino-5-*p*-chlorophenoxy-6-methylpyrimidine.

proguanil is only partially reversed by that compound. Further, although the antimalarial action of sulphonamides is readily inhibited by *p*-A.B. (Maier & Riley, 1942; Marshall, Litchfield & White, 1942; Bishop & McConnachie, 1948, 1951; Greenberg, 1949*c*; Thurston, 1950), the action of proguanil upon *P. gallinaceum* was found by Bishop & McConnachie (1948) to be unaffected by it; though Thurston (1950) found that in *P. berghei* its action is inhibited by *p*-A.B. and not by P.G.A.

Further evidence of the relationship between the mode of action of proguanil and folic acid antagonists comes from experiments with drug-resistant strains of malaria. Strains of *P. gallinaceum* treated with sulphadiazine or sulphanilamide became resistant not only to those drugs, but to proguanil also (Bishop & McConnachie, 1948, 1950*a*, *b*), an observation confirmed by Rollo (1951) and Thurston (1953*b*) on sulphadiazine-resistant strains of *P. berghei*. Although a strain of *P. gallinaceum* made resistant to proguanil was found by Bishop & McConnachie (1948) to be resistant to sulphadiazine, attempts to confirm this result have failed, nor have other workers reported cross-resistance to sulphonamides in their proguanil-resistant strains of malaria. Although in some experiments (Rollo, 1952), proguanil-resistant strains of *P. gallinaceum* and *P. cynomolgi* showed little enhanced resistance to pyrimethamine, in others cross-resistance appears to be reciprocal (Robertson, Davey & Fairley, 1952; Singh, Ray, Basu & Nair, 1952; Schmidt & Genther, 1952; Jones, 1953; Thurston, 1953).

Several synthetic pterins, related structurally to the pteridine portion of the folic acid molecule, but differing from it by the replacement of the OH group at position 4 by an amino group, were found by Daniel, Norris, Scott & Heuser (1947) to inhibit the growth of *Lactobacillus casei*, *L. arabinosus* and *Streptococcus faecalis*. Their anti-bacterial action was proved to be due to their antagonistic action towards pteroylglutamic acid. One of these compounds, 2:4-diamino-6:7-diphenylpteridine (0/63), was found by Greenberg (1949*a*) to be effective against *Plasmodium gallinaceum*, its activity being approximately equal to that of quinine.

A number of new synthetic 2:4-diaminopteridines, with various substituents in the 6:7 position of the molecule, synthesized by Campbell, Dunsmuir & Fitzgerald (1950), were found by Collier & Waterhouse (1950*a*) to show marked vibriostatic activity when tested *in vitro*. One of these compounds, 2:4-diamino-6:7-di*iso*-propylpteridine (0/129), was tested for antimalarial activity against *P. gallinaceum* by McConnachie (1953) and found to have a chemotherapeutic index of 1:40, about 3 times as great as that of quinine when tested by the same method. When 0/129 was tested in young chicks against a strain of *P. gallinaceum* made resistant to proguanil by treatment with that drug, and a sulphadiazine-proguanil-resistant strain made resistant by treatment with sulphadiazine, no cross-resistance was observed, indeed McConnachie found that both these strains were slightly hypersensitive to 0/129. In *P. berghei*, however, Thurston (1953) found that a sulphadiazine-resistant strain was cross-resistant to both proguanil and 0/129. Greenberg & Richeson (1951) found that their proguanil-resistant strain of *P. gallinaceum* was resistant to 2:4-diamino-6:7-diphenylpteridine (0/63), an observation confirmed by McConnachie on her proguanil- and sulphadiazine-proguanil-resistant strains.

A strain of *P. gallinaceum* resistant to 0/63 and two strains resistant to 0/129 have been developed. The relationship of these compounds to other antimalarials whose action is believed to be concerned with folic acid metabolism has been studied by cross-resistance tests in order to obtain further information upon their relationship. In addition, the effect of *p*-A.B. and P.G.A. upon 0/129, proguanil and sulphadiazine has been compared.

II. METHODS

The strains of *P. gallinaceum* used in the following experiments were maintained by intravenous injection of heparinized blood into young chicks of the domestic fowl, 45–65 g. in weight. The parent strain from which the 0/63 and 0/129-resistant strains were derived had, at no time in its history, been in contact with any drug and was maintained in a state of acute infection by passage through chicks at intervals of 2–4 days.

The drugs were given orally, by catheter tube into the stomach, with the exception that P.G.A. was, in one experiment, injected into the pectoral muscle, and *p*-A.B. in one experiment was given as the dry substance in gelatine capsules, whilst in another, the oral dose of *p*-A.B. solution was reinforced by the addition of a 1% mixture of the dry substance with the mash.

With doses of *p*-A.B. too great to be soluble in the required amount of water, solutions were obtained by the addition of sodium hydroxide. 0/129 base, 0/63 base and sulphadiazine were given as suspensions in 10% gum acacia. As neither pyrimethamine base nor 48–210 is very soluble in water, lactic acid was added in order to obtain solutions of these compounds. The drugs were given twice daily, morning and evening, the dose being proportional to the body weight of bird and expressed in mg./20 g. of bird.

The sensitivity of the drug-resistant and normal strains to other drugs was assessed by the method described previously (Bishop & Birkett, 1948).

In attempting to obtain a strain of malaria resistant to a drug, our practice has been to grade the size of the inocula and the doses of drug so that a strain which is being passed through the drug-treated birds at intervals of 2–4 days is maintained in a state of acute infection throughout the period of treatment. Large numbers of actively multiplying parasites are thus exposed to the action of the drug. The early doses to which the strain is exposed, therefore, should be slightly below the minimum dose effective upon the parent strain, that is to say, a dose which has some effect upon the parasites, but is not sufficient to inhibit the development of the infection drastically. As the minimum effective dose (i.e. the dose of drug which produced a 90% reduction of parasitaemia) of 0/129 upon *P. gallinaceum*, as assessed by McConnachie (1953), is 0·063 mg. twice daily, a dose of 0·025 mg. was chosen for the early treatment of *strain A*. The dose was gradually increased until after 8 months' treatment the strain was growing well in birds receiving 1·0 mg. twice daily. This dose is below the maximum (2·5 mg.) tolerated, but the supply of drug did not permit of further increase; there was, however, no indication that 1·0 mg. was the maximum dose to which resistance could develop, nor must it be assumed that the time taken to produce this degree of resistance is the

minimum required, since no effort was made to obtain resistance in the shortest possible period.

Strain B was treated in a similar way to *strain A* except that the dose to which the strain was first exposed was 0·05 mg. and that resistance was not developed beyond the 0·4 mg. dose level.

The initial dose of 0/63 used in preparing the 0/63-resistant strain was 0·64 mg. twice daily.

III. THE SENSITIVITY OF THE 0/129-RESISTANT STRAINS TO OTHER ANTIMALARIAL COMPOUNDS

After *strain A* had been maintained in birds treated with 1·0 mg. doses of 0/129 twice daily during twenty-seven passages through chicks at 3- or 4-day intervals, its growth in birds treated with this dose of drug was compared with its growth in untreated birds and found to be similar. Its sensitivity to other antimalarials was then assessed (Table 1). The strain was first tested against these compounds in

Table 1. *Comparison of the reaction of strain A, resistant to* 1·0 *mg.* 0/129, *and the parent strain of* Plasmodium gallinaceum *to sulphadiazine, proguanil, pyrimethamine and* 0/63

			Sulpha-diazine		Proguanil			Pyrimethamine						0/63		
Strain	Control	0/129* 1·0	0·8	0·6	1·0	0·5	0·1	0·1	0·01	0·005	0·002	0·001	0·0004	1·28	0·64	0·32
0/129A	309	273	49	—	—	—	332	—	—	—	—	—	—	—	—	—
parent	217	0	1	—	—	—	0	—	—	—	—	—	—	—	—	—
0/129A	315	—	—	54	1	—	—	—	—	—	—	—	—	—	—	—
parent	345	—	—	94	0	—	—	—	—	—	—	—	—	—	—	—
0/129A	354	—	—	—	95	284	260	—	—	—	—	—	—	—	—	—
parent	199	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—
0/129A	233	187	—	—	1	—	150	—	—	—	—	205	215	—	—	—
parent	340	0	—	—	—	—	2	—	—	—	—	17	269	—	—	—
0/129A	288	354	—	—	—	—	—	—	55	—	340	410	—	—	—	—
parent	349	0	—	—	—	—	—	—	—	—	1	—	—	—	—	—
0/129A	302	—	—	—	—	—	—	—	108	199	314	—	—	—	257	247
parent	322	—	—	—	—	—	—	—	0	—	0	—	—	—	5	248
0/129A	201	—	12	62	61	307	—	0	50	—	—	—	—	260	244	—
parent	323	—	6	10	—	—	1	—	0	—	—	—	—	—	51	—
0/129A	295	—	1	2	9	—	—	—	12	130	—	—	—	—	—	—
parent	352	—	1	6	—	—	1	—	—	—	—	0	—	—	—	—

* Throughout the tables, the dose, unless stated otherwise, was given orally twice daily for 3½ days, and is expressed in mg./20 g. of body weight of bird. The intensity of infection is expressed as the average number of infected erythrocytes per 500 erythrocytes in groups of four to six birds.

doses around the minimum effective level, but if it proved resistant to them it was tested against higher doses. No cross-resistance to sulphadiazine was demonstrated. Good growth was obtained in birds treated with 0·1 and 0·5 mg. of proguanil, but in birds treated with 1·0 mg. it was variable—in a few birds moderately heavy infections were produced, whereas in others no growth occurred.

In birds treated with pyrimethamine in doses slightly above the minimum effective level, growth was heavy, but with higher doses there was greater variability in the intensity of infection, and in birds treated with 0·1 mg. no growth occurred. In birds treated with 0/63 growth occurred over the small range of doses tested.

When strains of *P. gallinaceum* were treated with sulphadiazine (Bishop & McConnachie, 1950*a*) and sulphanilamide (Bishop & McConnachie, 1950*b*), the strains became resistant not only to the sulphonamides, but to proguanil also. The degree of resistance to proguanil produced by these sulphonamides, however, did not depend upon the degree of resistance to the sulphonamides themselves; thus, full resistance to proguanil was produced in a strain treated with the minimum effective dose of sulphadiazine (0·625 mg.) for only 17 days though no enhancement of resistance to sulphadiazine could as yet be detected, and in the sulphanilamide-resistant strain resistance to proguanil was complete over the range of doses tolerated though sulphanilamide is a drug of much lower antimalarial potency than proguanil. From the results of cross-resistance tests upon *strain A* recorded in Table 1 it is obvious that prolonged treatment with relatively large doses of 0/129 does not produce full resistance to proguanil or pyrimethamine.

Table 2. *Comparison of the reaction of strain B resistant to* 0/129 *and the parent strain of* Plasmodium gallinaceum *to sulphadiazine, proguanil, pyrimethamine and* 48–210.

Strain	No. of passages through 0/129	Control	0/129			Sulphadiazine		Proguanil			Pyrimethamine			48–210	
			0·4	0·2	0·1	0·8	0·6	1·0	0·5	0·1	0·005	0·002	0·001	0·2	0·1
0/129B	10	228	—	—	156	—	—	—	1	34	—	—	—	—	—
parent	—	221	—	—	0	—	—	—	—	0	—	—	—	—	—
0/129B	12	272	—	—	151	—	—	—	—	90	—	—	—	—	—
parent	—	349	—	—	6	—	—	—	—	0	—	—	—	—	—
0/129B	22	269	30	206	—	—	—	25	97	167	0	0	5	—	—
parent	—	305	—	0	—	—	—	—	—	9	—	0	10	—	—
0/129B	31	240	193	—	—	—	—	96	196	237	118	—	291	—	—
parent	—	344	0	—	—	—	—	—	—	0	—	—	39	—	—
0/129B	34	284	—	—	—	0	0	—	—	—	16	—	—	254	152
parent	—	395	—	—	—	2	7	—	—	—	1	—	—	0	14
0/129B	36	418	—	—	—	—	—	—	—	—	44	320	—	—	—
parent	—	393	—	—	—	—	—	—	—	—	0	1	—	—	—

In order to find out whether resistance to these drugs develops concurrently with resistance to 0/129 in birds treated with that drug, a new substrain, *strain B*, was treated with gradually increasing doses of 0/129 and tested for cross-resistance as soon as a low grade resistance to 0/129 had developed. After being passaged 10 times over a period of 56 days, through birds receiving 0·05 mg. of the drug in the first passage, rising to 0·2 mg. doses in the 8th, 9th and 10th passages, the strain was found to produce rather light infections of parasites of normal appearance in birds treated with 0·1 mg. doses of 0/129 and poor infections of abnormal parasites in birds receiving 0·1 mg. doses of proguanil (Table 2). Two further serial passages

of the strain were made through birds treated with 0·2 mg. doses of 0/129 twice daily after which somewhat heavier infections of parasites, normal in appearance, were produced in birds treated with 0·1 mg. doses of proguanil. After ten further serial passages through birds treated with 0·4 mg. doses of 0/129, making a total of twenty-two passages through treated birds, the strain showed an increase in resistance to proguanil, but no resistance to pyrimethamine could be detected. After nine more passages through birds receiving 0·4 mg. doses of 0/129, resistance to proguanil had again increased and some resistance to pyrimethamine was found to have developed; but even after 5½ months' treatment with 0·4 mg. doses of 0/129 resistance extended only to the lower effective doses of pyrimethamine. This strain was also tested for cross-resistance to 48–210, a member of the phenoxy series of the 2:4-diaminopyrimidines (Rollo, 1952), and showed resistance to the doses tested. As in *strain A*, no resistance to sulphadiazine could be detected.

Table 3. *The effect of pyrimethamine upon a proguanil-resistant strain of* Plasmodium gallinaceum

	Strains					
	Prog.	Par.	Prog.	Par.	Prog.	Par.
Controls	220	203	302	229	280	373
Proguanil						
0·1	145	0	—	0	—	—
0·5	162	—	276	—	326	0
1·0	—	—	13	—	235	—
1·5	—	—	—	—	115	—
Pyrimethamine						
0·0005	162	67	—	—	—	—
0·001	151	5	247	1	—	—
0·005	—	—	310	—	—	—
0·01	—	—	138	—	205	1
0·05	—	—	1	—	285	—
0·1	—	—	—	—	211	—
0·5	—	—	—	—	12	—

Prog. = Proguanil-resistant strain. Par. = Parent strain.
Intensity of infection expressed as the average number of parasitized erythrocytes per 500 erythrocytes, in groups of three to five birds.

For comparison with the 0/129-resistant strains, the reaction of a proguanil-resistant strain of *P. gallinaceum* to pyrimethamine was studied. When first tested, this strain was not fully resistant to proguanil (Table 3), but it showed a 10 to 20-fold enhancement of resistance to pyrimethamine. After further treatment with proguanil the strain became fully resistant to that drug, producing infections of normal parasites in birds treated with the maximum tolerated dose of the drug (1·5 mg.). A further 10-fold increase in resistance to pyrimethamine developed concurrently.

IV. THE SENSITIVITY OF AN 0/63-RESISTANT STRAIN TO OTHER ANTIMALARIAL COMPOUNDS

A substrain of the parent strain was treated with 0/63 in twice daily doses increasing from 0·64 to 2·56 mg. over a period of 2 months. When tested for cross-resistance to low, but effective doses of proguanil, pyrimethamine, 48–210 and 0/129 it proved to be resistant. Like the 0/129-resistant strains, its sensitivity to sulphadiazine was unchanged (Table 4).

Table 4. *The effect of proguanil, sulphadiazine, pyrimethamine and* 2:4-*diamino*-6:7-*diisopropylpteridine* (0/129) *upon a* 2:4-*diamino*-6:7-*diphenylpteridine* (0/63)-*resistant strain of* Plasmodium gallinaceum

		Strains					
	Dose	0/63	Par.	0/63	Par.	0/63	Par.
Controls		359	360	414	356	340	329
0/63	1·28	310	0	—	—	233	0
Proguanil	0·1	218	0	—	—	—	—
Sulphadiazine	0·6	—	—	0	1	—	—
Sulphadiazine	0·8	0	0	—	—	—	—
Pyrimethamine	0·001	—	—	368	0	—	—
0/129	0·1	—	—	411	0	—	—
48–210	0·2	—	—	—	—	182	1
48–210	0·1	—	—	—	—	265	18

0/63 = 0/63-resistant strain. Par. = Parent strain.
Intensity of infection expressed as the average number of parasitized erythrocytes per 500 erythrocytes, in groups of three to five birds.

V. A COMPARISON OF THE EFFECT OF *p*-AMINOBENZOIC ACID (*p*-A.B.) AND PTEROYLGLUTAMIC ACID (P.G.A.) UPON SULPHADIAZINE, PROGUANIL AND 2:4-DIAMINO-6:7-DI*ISO*PROPYLPTERIDINE (0/129)

Since the 0/129-resistant strain of *P. gallinaceum* showed cross-resistance to proguanil and not to sulphadiazine, the effect of *p*-A.B. and P.G.A. upon these compounds was compared using the parent strain of *P. gallinaceum* for the tests. The dose of sulphadiazine used in the initial experiments was 0·8 mg. This dose, when given twice daily from the day of inoculation for 3½ days, suppressed infections induced by an inoculum of 50 million parasites to a low level, but did not inhibit them completely. Its action was completely antagonized by 0·08 mg. doses of *p*-A.B. (Table 5, Expts. 2 and 3). In previous experiments (Bishop & McConnachie, 1951) the inhibition of the antimalarial action of sulphadiazine by *p*-A.B. was shown to be competitive, since 5 and 2·5 mg. doses of sulphadiazine were antagonized by $\frac{1}{10}$th the amount of *p*-A.B. and partially antagonized by $\frac{1}{100}$th the amount.

P.G.A., when given orally in doses of 0·08 mg., failed to antagonize the action of 0·8 mg. of sulphadiazine (Table 5, Expts. 2, 4 and 6), but in one experiment (Table 5, Expt. 1), in which P.G.A. was given intramuscularly, the action of the sulphadiazine was completely antagonized in some chicks, but not in others. With higher doses of P.G.A. (0·2 mg. and upwards, Table 5, Expts. 5, 7 and 8) the

antagonistic action of this compound was complete and consistent in all the chicks tested. With 4·0 mg. doses of sulphadiazine (Table 5, Expt. 8) antagonism was slight even when the dose of P.G.A. equalled that of the sulphadiazine. Unlike *p*-A.B., therefore, the antagonistic action of P.G.A. is not competitive. In some bacteria P.G.A. acts as a non-competitive antagonist of sulphonamides—a relatively constant amount of the vitamin antagonizing any level of the inhibitor (Lampden & Jones, 1946*a*). Its action upon *P. gallinaceum*, however, does not conform to this pattern, since when 1 mg. doses of P.G.A., which antagonize the action of 0·8 mg. of sulphadiazine upon *P. gallinaceum* completely, were tested against doses of sulphadiazine ranging from 1·5 to 10 mg., their antagonistic action declined as the dose of sulphadiazine increased (Table 5, Expt. 7).

Table 5. *The effect of* p-*aminobenzoic acid* (p-*A.B.*) *and pteroylglutamic acid* (*P.G.A.*) *upon the action of sulphadiazine* (*S.D.*) *upon* Plasmodium gallinaceum

	Expt. no.							
	1	2	3	4	5	6	7	8
Controls	413	278	279	395	393	377	365	330
S.D. 0·8	9	7	12	2	3	28	6	9
S.D. 1·5	—	—	—	—	—	—	2	—
S.D. 4·0	—	—	—	—	—	—	—	5
P.G.A. 0·08	384	230	—	—	—	334	—	—
P.G.A. 1·0	—	—	—	—	—	—	280	—
P.G.A. 4·0	—	—	—	—	—	—	—	261
p-A.B. 0·08	—	273	330	—	—	—	—	—
S.D. 0·8 + P.G.A. 0·08	290	11	—	12	—	38	—	—
S.D. 0·8 + P.G.A. 0·2	—	—	—	—	—	—	—	275
S.D. 0·8 + P.G.A. 0·6	—	—	—	—	—	—	—	208
S.D. 0·8 + P.G.A. 1·0	—	—	—	—	340	—	346	291
S.D. 0·8 + P.G.A. 6·0	—	—	—	—	401	—	—	—
S.D. 4·0 + P.G.A. 0·4	—	—	—	—	—	—	—	52
S.D. 4·0 + P.G.A. 1·0	—	—	—	—	—	—	—	59
S.D. 4·0 + P.G.A. 4·0	—	—	—	—	—	—	—	102
S.D. 1·5 + P.G.A. 1·0	—	—	—	—	—	—	125	—
S.D. 2·5 + P.G.A. 1·0	—	—	—	—	—	—	75	—
S.D. 5·0 + P.G.A. 1·0	—	—	—	—	—	—	62	—
S.D. 10·0 + P.G.A. 1·0	—	—	—	—	—	—	44	—
S.D. 0·8 + *p*-A.B. 0·08	—	230	260	—	—	—	—	—

Intensity of infection expressed as the average number of parasitized erythroyctes per 500 erythrocytes, in groups of three to five birds.

Whereas, formerly, Bishop & McConnachie (1948) and Greenberg (1949*c*) found that *p*-A.B. did not reverse the action of proguanil upon *P. gallinaceum*, the latter (Greenberg, 1953) has recently described a reversal of the action of 0·04 mg./20 g. of proguanil with 4 mg./20 g. of *p*-A.B. equal to that produced by a similar dose of folic acid. The degree of reversal obtained was, however, of a very low order.

The effect of *p*-A.B. and P.G.A. upon proguanil has been reinvestigated. A dose of proguanil (0·04 mg. twice daily), similar to that used by Greenberg, suppressed

parasitaemia to a low level, but did not inhibit the development of parasites completely. 4·0 mg. doses of *p*-A.B., given in capsules as in Greenberg's experiments, did not reverse the antimalarial action of the proguanil (Table 6, Expt. 7). With 10 mg. of *p*-A.B., a dose 250 times that of proguanil, again no reversal occurred, though in one experiment (Table 6, Expt. 6) the 10 mg. doses of *p*-A.B. were reinforced by 1 % *p*-A.B. in the diet in order to obtain a more continuous intake of that factor, since Thurston (1954) has found that, in *P. berghei*, the antagonistic action of this compound is greater when imbibed frequently, as a solution in the drinking water, by the host. In chickens treated with 25 mg. doses of *p*-A.B. the infections were of a low order and the antimalarial action of 0·04 mg. doses of proguanil was not affected by this dose (Table 6, Expt. 5). An inhibitory effect by *p*-A.B., when given in large doses, was first observed by Marshall, *et al.* (1942) in *P. lophurae*.

When 0·4 mg. doses of P.G.A. were given with 0·04 mg. doses of proguanil (Table 6, Expt. 3) no reversal of the action of proguanil occurred, but with 2·0 mg. doses of P.G.A. (Table 6, Expt. 2) the infections, though not so heavy as in the

Table 6. *The effect of pteroylglutamic acid* (*P.G.A.*) *and* p-*aminobenzoic acid* (p-*A.B.*) *upon the action of* 2:4-*diamino*-6:7-*diisopropylpteridine* (0/129) *and proguanil* (*Prog.*) *upon* Plasmodium gallinaceum

	Expt. no.								
	1	2	3	4	5	6	7	8	9
Controls	221	385	207	352	353	305	395	377	341
0/129 0·063	—	—	—	25	3	—	11	—	43
0/129 0·08	1	—	—	—	—	—	—	0	—
Prog. 0·04	—	69	6	—	16	20	10	—	—
P.G.A. 0·4	—	—	290	—	—	—	—	—	—
P.G.A. 0·8	140	—	—	—	—	—	—	—	—
P.G.A. 1·0	—	—	—	—	—	—	—	—	376
P.G.A. 2·0	—	364	—	—	—	—	—	—	—
p-A.B. 4·0	—	—	—	—	—	—	329*	—	—
p-A.B. 10	—	—	—	—	236	235†	—	—	—
p-A.B. 25	—	—	—	—	39	—	—	—	—
0/129 0·063 + P.G.A. 0·5	—	—	—	—	—	—	—	—	46
0/129 0·063 + P.G.A. 1·0	—	—	—	—	—	—	—	—	78
0/129 0·063 + P.G.A. 2·0	—	—	—	—	254	—	—	—	—
0/129 0·063 + P.G.A. 6·0	—	—	—	361	—	—	—	—	—
0/129 0·08 + P.G.A. 0·8	15	—	—	—	—	—	—	—	—
0/129 0·08 + P.G.A. 8·0	—	—	—	—	—	—	—	38	—
0/129 0·063 + *p*-A.B. 4·0	—	—	—	—	—	—	22*	—	—
0/129 0·063 + *p*-A.B. 10·0	—	—	—	—	12	—	—	—	—
0/129 0·063 + *p*-A.B. 25·0	—	—	—	—	25	—	—	—	—
Prog. 0·04 + P.G.A. 0·4	—	—	48	—	—	—	—	—	—
Prog. 0·04 + P.G.A. 2·0	—	247	—	—	—	—	—	—	—
Prog. 0·04 + *p*-A.B. 4·0	—	—	—	—	—	—	5*	—	—
Prog. 0·04 + *p*-A.B. 10·0	—	—	—	—	3	32†	—	—	—
Prog. 0·04 + *p*-A.B. 25·0	—	—	—	—	7	—	—	—	—

* *p*-A.B. administered as dry substance in gelatine capsules.
† 1 % *p*-A.B. in diet in addition to oral dose.

controls of that particular experiment, were within the range of intensity of infection of the controls in the experimental series as a whole, and much heavier than those obtained by Greenberg with 4·0 mg. doses.

The antimalarial action of 0·08 mg. doses of 0/129 was not reversed by P.G.A. in doses 10 to 100 times greater than those of 0/129 (Table 6, Expts. 1 and 8); but when slightly smaller, though still inhibitory doses of 0/129 (0·063 mg.) were used, complete reversal of its action occurred with 2·0 and 6·0 mg. doses of P.G.A. (Table 6, Expts. 4 and 5), but not with 4, 10 or 25 mg. doses of *p*-A.B. (Table 6, Expts. 5 and 7).

VI. DISCUSSION

The effect of *p*-A.B., folic and folinic acids upon the antimalarial action of certain pteridines, pyrimethamine and related compounds has been studied in several species of bacteria. Collier & Waterhouse (1950*b*) found that the vibriostatic activity of 0/129 was slightly reduced by relatively large amounts of P.G.A., whereas *p*-A.B. had little effect, though it antagonized the vibriostatic action of sulphaguanidine.

Several 2:4-diaminopteridines, of which 0/63 was one, were found by Daniel, *et al.* (1947) to have the capacity of inhibiting the growth not only of bacteria which synthesize folic acid, but also of those which require the preformed vitamin. Their action is interpreted as preventing the use of folic acid. These compounds had, however, much less effect upon *Lactobacillus casei* than upon *Streptococcus faecalis*, although both organisms require approximately the same amount of preformed folic acid, which suggests that more than one system may be involved. Thus *Lactobacillus casei* may possess several separate systems concerned with folic acid metabolism, of which one is affected by the pterins.

Pyrimethamine has been shown to be a competitive antagonist of folic and folinic acids in *L. casei* and of folinic acid (citrovorum factor) in *Leuconostoc citrovorum*, but the range over which the competitive inhibition occurs is more restricted in the latter species than in the former. With *Streptococcus faecalis*, which grows equally well with folic and folinic acids, pyrimethamine has a more pronounced action against folic than folinic acid. It is uncertain whether the metabolite with which pyrimethamine and the related pyrimidines are competing is identical with synthetic folinic acid, since both liver powder and autolysed yeast have greater antagonistic effects than would be expected from their folinic acid content (Hitchings, Falco, Vanderwerff, Russell & Elion, 1952).

In several species of bacteria sensitive to the action of sulphonamides, the inhibitory action of these drugs is antagonized competitively by *p*-A.B. and non-competitively by P.G.A. (Lampden & Jones, 1946*b*). Their action has been interpreted as inhibiting the synthesis of folic acid via *p*-A.B. (Lampden & Jones, 1946*a*).

It is not profitable to compare the *in vitro* action of proguanil upon bacteria with its *in vivo* action upon the malaria parasite, since it is now known that its antimalarial action is exerted in the form of a metabolized product (Carrington, Crowther, Davey, Levi & Rose, 1951; Crowther & Levi, 1953).

The results of the experiments recorded above have shown that the action of 0/129 upon *Plasmodium gallinaceum* is unaffected by *p*-A.B. and inhibited only in the minimum effective dose by a relatively large amount of P.G.A. In this the action of 0/129 upon *P. gallinaceum* resembles its vibriostatic action. Similar

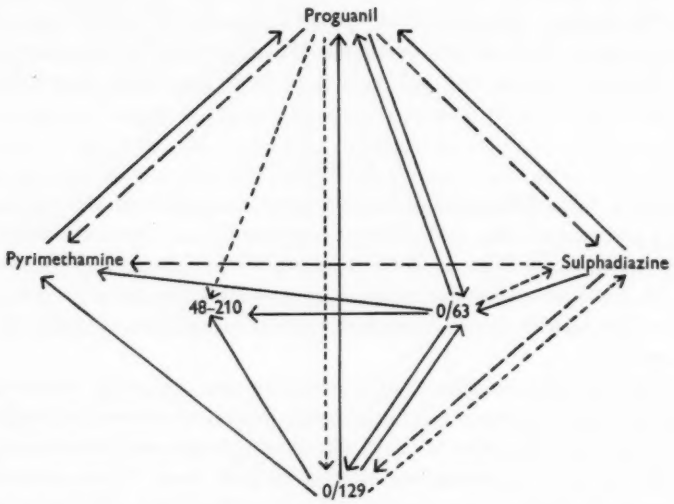
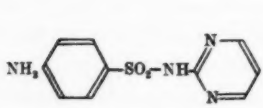
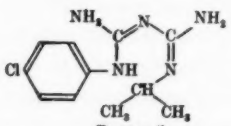
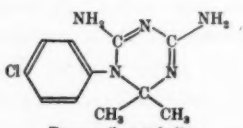
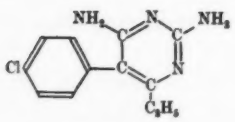


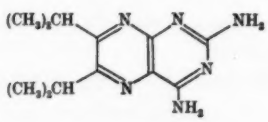
Fig. 1. Diagram showing cross-resistance between proguanil, pyrimethamine, sulphadiazine, 0/129, 0/63, and 48–210, in various strains and species of malaria, derived from all available data. $X \longrightarrow Y$, a strain resistant to X is cross-resistant to Y; $X \cdots\cdots \rightarrow Y$, a strain resistant to X has been tested for cross-resistance to Y, but no cross-resistance occurs; $X - - - - \rightarrow Y$, strains resistant to X have been tested for cross-resistance to Y, but the results were variable.

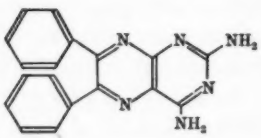

Sulphadiazine


Proguanil


Proguanil metabolite


Pyrimethamine


0/129


0/63

results were obtained with proguanil with *P. gallinaceum*, though in *P. berghei* Thurston (1950) has found its action and that of pyrimethamine and some 2:4-diaminopteridines (Thurston, 1953*a*) to be inhibited by *p*-A.B. and not by P.G.A. The action of 0/63 upon *P. gallinaceum* was shown by Greenberg (1949*a*) to be inhibited significantly, but not completely, by P.G.A., but he gives no data of the

effect of *p*-A.B. upon this compound. Similar results were obtained with 2:4-diamino-5-(*p*-chlorophenoxy)-6-methylpyrimidine and 2:4-diamino-5-*p*-chlorophenoxypyrimidine (Greenberg & Richeson, 1950). Rollo (Hitchings, 1952), however, failed to reverse the action of pyrimethamine upon *P. gallinaceum* with folinic acid.

The relationship of the antimalarial action of these compounds to the folic acid system, therefore, resembles fairly closely their antibacterial action. In their action upon *P. gallinaceum* these compounds differ from sulphadiazine, and presumably related sulphonamides, since the antimalarial action of sulphadiazine is antagonized competitively by *p*-A.B. The results of the cross-resistance tests confirm this difference (Fig. 1). The 0/129 and 0/63-resistant strains of *P. gallinaceum* were not resistant to sulphadiazine, though they were cross-resistant to each other and to proguanil, 48–210 and pyrimethamine. Results of experiments upon synergism between proguanil, 48–210, and 0/63 and sulphadiazine also demonstrated this difference. Thus the antimalarial action of proguanil (Greenberg, Boyd & Josephson, 1948), 0/63 (Greenberg, 1949*a*) and 48–210 (Greenberg & Richeson, 1950) was potentiated by sulphadiazine, whereas proguanil did not synergize with 48–210 (Greenberg & Richeson, 1951).

However, there is some evidence that resistance to proguanil, pyrimethamine, 0/129 and 0/63 may not develop by identical pathways since in *P. gallinaceum* a sulphadiazine-resistant strain, though resistant to proguanil and 0/63, was not resistant to 0/129, nor was a proguanil-resistant strain resistant to either sulphadiazine or 0/129 (McConnachie, 1953), whereas a sulphadiazine-resistant strain of *P. berghei* was resistant to both proguanil, 0/129 and pyrimethamine (Thurston, 1953*b*). Rollo (1951), however, found no cross-resistance to pyrimethamine in a sulphadiazine-proguanil-resistant strain of *P. berghei*. The difference in the effect of *p*-A.B. upon the action of proguanil in these two species has already been commented upon. Also, a proguanil-resistant strain of *P. gallinaceum*, which was cross-resistant to 0/63, was not cross-resistant to 2:4-diamino-5-(*p*-chlorophenoxy)-6-methylpyrimidine (48–210) (Greenberg & Richeson, 1951), though, in the experiments recorded above, the 0/129-resistant *strain B* was resistant to this compound as well as to 0/63, and the 0/63-resistant strain was resistant to 48–210. Falco, Goodwin, Hitchings, Rollo & Russell (1951) found that proguanil-resistant strains of both *P. gallinaceum* and *P. berghei* were not resistant to 2:4-diamino-5-(*p*-chlorophenoxy)-6-methylpyrimidine. Moreover, in a strain of *P. gallinaceum* treated with 0/129 the development of resistance to that compound itself preceded resistance to proguanil, and resistance to proguanil preceded resistance to pyrimethamine.

SUMMARY

1. Two strains of *Plasmodium gallinaceum* were made resistant to 2:4-diamino-6:7-di*iso*propylpteridine (0/129) by treatment with that drug.

2. The 0/129-resistant strains were resistant to proguanil, pyrimethamine, 2:4-diamino-6:7-diphenylpteridine (0/63) and 2:4-diamino-5-(*p*-chlorophenoxy)-6-methylpyrimidine (48–210), but not to sulphadiazine.

3. In one strain treated with 0/129, the development of resistance to that drug itself preceded resistance to proguanil, and resistance to proguanil preceded resistance to pyrimethamine.

4. A strain of *P. gallinaceum* made resistant to 0/63 was resistant to proguanil, pyrimethamine and 0/129, but not to sulphadiazine.

5. The action of 0/129 and proguanil upon *P. gallinaceum* was not antagonized by *p*-A.B., though in the minimum effective dose their action was antagonized by relatively large doses of P.G.A.

6. Whereas the action of sulphadiazine upon *P. gallinaceum* was antagonized competitively by *p*-A.B., it was antagonized by P.G.A. only when the sulphadiazine was given in small doses.

I am indebted to Dr H. O. J. Collier, and Messrs Allen and Hanbury Ltd., for the supply of 0/129 and 0/63; to Dr I. M. Rollo of the Wellcome Laboratories of Tropical Medicine for the pyrimethamine; to Dr G. H. Hitchings of the Wellcome Research Laboratories Tuckahoe, U.S.A., for the 48–210, to Dr D. G. Davey and the Imperial Chemical (Pharmaceuticals) Ltd. for the proguanil, and to Dr H. P. Broquist and the American Cyanamid Co. for the pteroylglutamic acid.

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NEMATODE PARASITE POPULATIONS IN SHEEP ON LOWLAND FARMS

I. WORM EGG COUNTS IN EWES

By H. D. CROFTON, B.Sc., Ph.D.

Department of Zoology, University of Bristol

(With 6 Figures in the Text)

I. INTRODUCTION

Several workers have studied the seasonal fluctuation of worm egg counts in sheep. Taylor (1935) described the results obtained by sampling twelve ewes monthly for more than 2 years. Counts were made on composite samples. The sheep were on a lowland pasture in the south of England. A detailed study of seasonal fluctuation on hill pastures has been made in Scotland by Morgan and his co-workers (Morgan & Sloan, 1947; Morgan, Parnell & Rayski, 1950, 1951; Wilson, Morgan, Parnell & Rayski, 1953). Cushnie & White (1948) have also described results obtained from sampling, Blackface sheep in Scotland. Hawkins, Cole, Kline & Drudge (1944), Hawkins & Freitas (1947) and Seghetti & Marsh (1945) have given records from sheep in the United States, and Naerland (1949) has given similar details of sheep in Norway. All of these workers have shown that although there is a relatively low worm egg count for ewes during most of the year, there is a marked increase during the spring. In some cases there are indications of a second increase during the summer.

The present work is most nearly comparable with that of Taylor (1935) in so far as pasture and climatic conditions are concerned but Taylor's use of composite samples taken at monthly intervals, resulted in loss of a considerable amount of information necessary for a proper interpretation of his figures.

II. MATERIALS AND METHODS

From May 1950 to August 1953 observations were made on ewes from normal commercial flocks in the south-west of England. Attention was mainly devoted to one flock in Somerset and two in Gloucestershire, but some observations were made on 'flying flocks' as opportunity occurred. The breeds of sheep on these farms were as follows: flock A (Somerset)—Cluns; flock B (Gloucestershire)—Suffolk, three-quarter Suffolk and Hampshire × Leicester cross-breds; flock C (Gloucestershire) Suffolk cross-bred and Hampshire cross-bred. Observations were not commenced on this latter flock until 1951 and ended in early 1953. All the sheep in each flock were marked by tattooing their ears, each sheep being given a separate number so that it could be identified individually. Samples of faeces were collected from the rectum of the sheep, each sample being marked with the number of the ewe and stored in a refrigerator until examined. In the first two years of the investigation each sample was split into two parts when sufficient

material was available; one, consisting of 2 g. of faeces, was used for egg counting; the other was used for culturing larvae for species determination. In the latter part of the investigation few cultures of larvae were made, an egg differentiation method being used (see Cunliffe & Crofton, 1953).

The McMaster technique was used for egg counting (see Gordon & Whitlock, 1939), six slide chambers being used for each count. Eggs of *Nematodirus* spp. and *Strongyloides* spp. were recorded separately, but no attempt was made to count eggs of *Trichurus*. When cultures were made the method used was similar to that employed in the McMaster Laboratories (Gordon, private communication), this being very suitable when only small amounts of faeces were available. After culture for 10–14 days the larvae were removed and iodine added. Differential counts were made either immediately or after storage. Usually 100 larvae were identified but in some cases, where the yield of larvae was low, only fifty larvae were examined. Throughout the investigation sheep in flock A received no anthelmintic treatment. Anthelmintic treatment was given occasionally to flock C and more regularly to flock B. In both cases the time of administration of phenothiazine or CuNic mixture is indicated in the diagrams, and in some cases remarked upon in the text.

III. RESULTS

The figures given in the diagrams are all calculated in the usual way and represent the mean number of eggs per gram per sheep. Detailed examination of the individual counts has shown that the *mean* is not necessarily the best estimate of flock infection and that the counts within a flock are not distributed normally. From the relatively small amount of data available the distribution seems to approach that of the negative binomial type, and a logarithmic or square root transformation would appear to be desirable. No transformation has been made for two reasons, first the concern of the work has been with general trends, and where this has been otherwise the individual figures are quoted. So far as can be seen neither a logarithmic nor a square root transformation would materially alter the general picture; secondly, the presentation of data as worm eggs per gram is almost universally adopted and any other method of presentation can be confusing because, while most workers realize that the number of worm eggs per gram is based on artificial units when population studies are attempted, they might, unconsciously, attribute greater significance to transformed data. While the usual method of presentation is adopted it is realized that transformation of the counts may be necessary for some other purposes in the future. Since this was written a valuable paper by Hunter & Quenouille (1952) has discussed worm egg counts and their interpretation more fully.

(*a*) *Worm egg counts* 1950–3

In most cases faeces samples were collected at fortnightly intervals, but at some periods sampling was weekly and at times samples were taken every second day. During lambing time the collection of samples was postponed because it was thought undesirable to pen and handle the ewes.

(i) *Flock A.*

This flock was not given any anthelmintic treatment. Fig. 1*a–d* shows the number of worm eggs per gram per sheep (eggs of *Nematodirus* and *Strongyloides* not included).

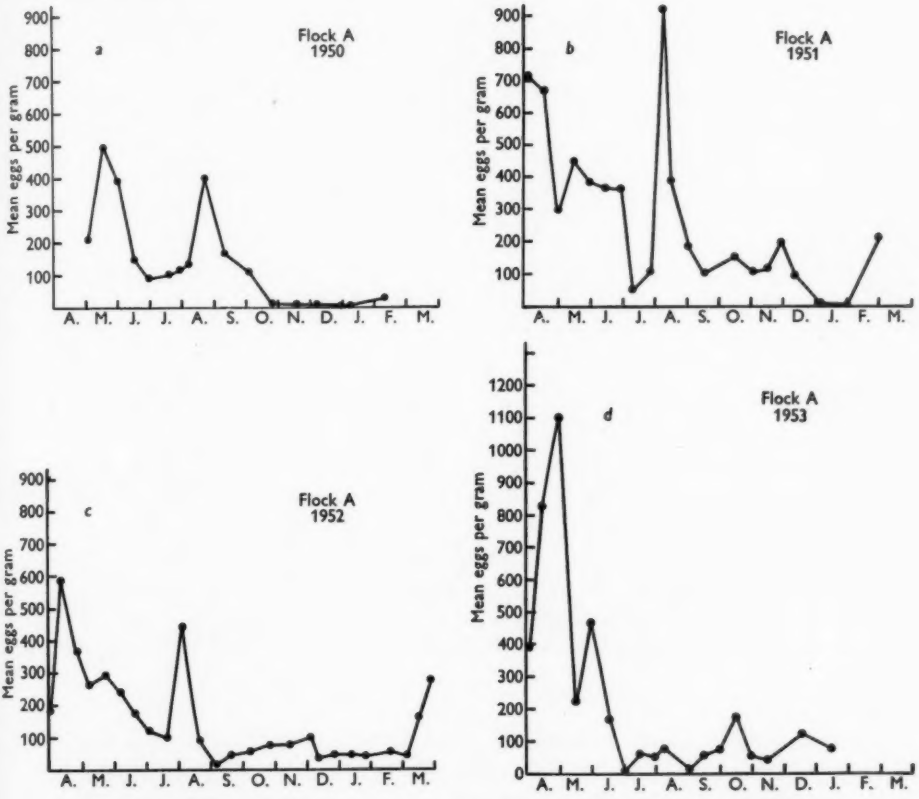

Fig. 1. *a–d.* The number of worm eggs per gram per sheep. Flock A, 1950–3.

The general level of infection of these ewes was low, and for most of the time the counts were below 200 e.p.g. In spring (April–May) of each year there was a rapid rise in egg counts. A similar rise occurred in August in each year. Fig. 2 shows the worm egg counts of some individual sheep. During spring it will be seen that the period of rise in egg count of individuals was relatively short, high counts being observed for a fortnight or less. This is also true of the increases occurring in August, but the reduction in counts was more gradual than in the earlier part of the year. Although the mean egg counts indicate that the spring and summer rises were similar, the rise in individual counts was greater in spring than in summer. This is a real difference, masked because the time of occurence of the spring rise in individual sheep varied, whereas in summer all the high counts occurred within a short period.

(ii) *Flock B*

Fig. 3*a–d* shows the number of eggs per gram per sheep during the period of the investigation. The times of anthelmintic treatment are marked on the diagrams. The general course of the infection is similar to that in flock A, although the counts are slightly lower in some cases. Regular anthelmintic treatment has not altered the general pattern of worm egg production but has lowered the counts. The

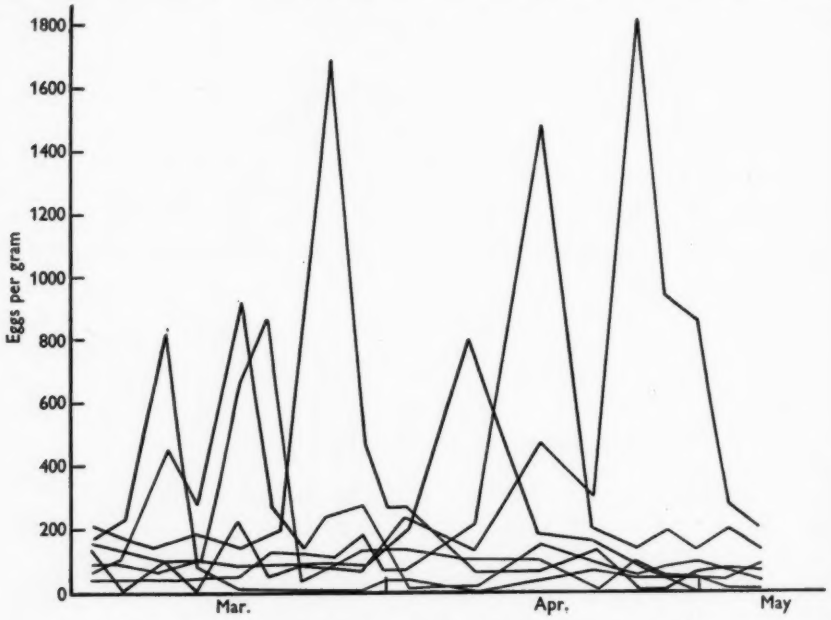

Fig. 2. Worm egg counts of individual sheep on Flock A.

difference between flock B and flock A figures is less than might have been expected between a dosed and an undosed flock. Diagrams showing the counts of individual sheep are not given because they are essentially similar to those for flock A.

(iii) *Flock C*

Fig. 4*a*, *b* shows the number of eggs per gram per sheep for the years 1951 to 1953. Observations on this flock were started a year later than those on flocks A and B and ended in spring 1953. Anthelmintic treatment was given occasionally and the times are marked on the graphs. The difference between flocks C, and B which was dosed regularly, is not great. Fig. 4*b* (1952–3) is not strictly comparable with Fig. 4*a* because the flock, while running as a unit, was divided for purposes of sampling into a number of subgroups arranged according to the time of lambing of the ewes. Ewes in group X lambed in the period 1–31 March those in group Y from 23 April to 28 May. Pooling of these results would give results comparable with Fig. 4*a*, but this has not been done here because the present arrangement illustrates an important point which will be considered later.

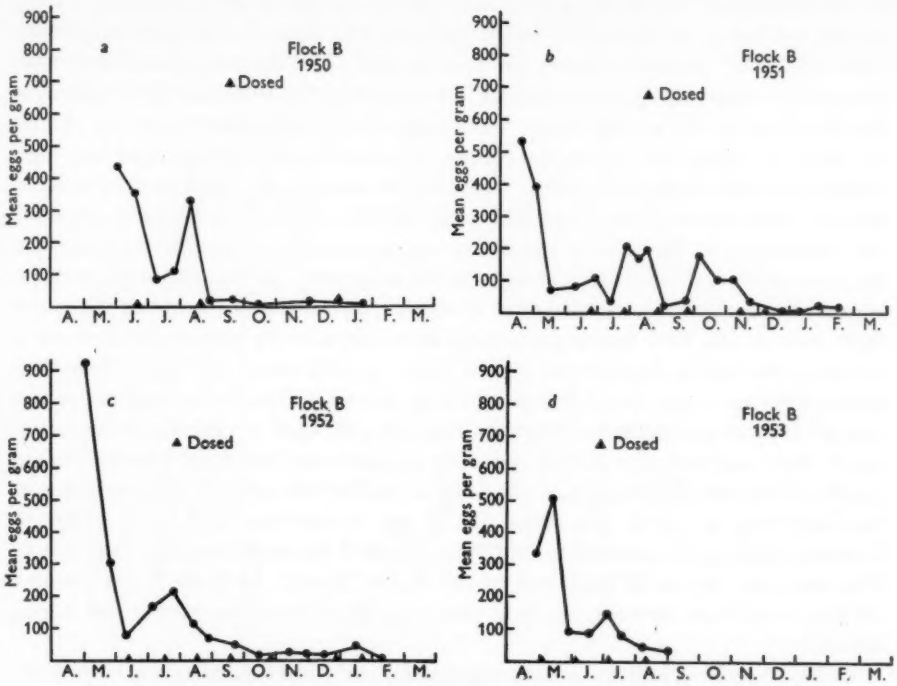

Fig. 3. *a–d*. The number of worm eggs per gram per sheep. Flock B, 1950–3.

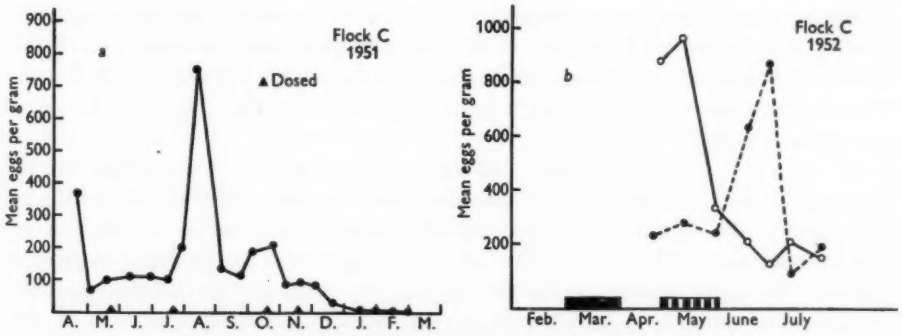

Fig. 4*a*, *b*. The number of worm eggs per gram per sheep. Flock C, 1951, 1952. (See text for Fig. 4*b*.)

(*b*) *Worm egg counts in relation to lambing time*

During 1950 the increase in egg counts in May and June in flocks A and B was similar to the 'spring rise' recorded by other workers. It was apparent after studying the records of individual sheep (see Fig. 2) that the general increase in egg counts was a result of increases in the egg counts of individuals, but that these increases were not simultaneous. In flock A, for example, the sheep were sampled

on three different occasions during May 1950, but individual ewes showed high counts on one or at the most, two occasions. The high counts were invariably followed by low counts in June. In flock B (1950) a similar thing occurred. Although the observations on this flock did not start until the beginning of June it is obvious that the initial high count was similar to the peak observed in the counts for flock A. Again the rise in the counts of individual sheep was rapid and high counts for each sheep was mainly restricted to one, at the most two, occasions. Similar observations were made each year, and for each of the flocks throughout the investigation. The time of the rise in egg counts differed in different years for the same flock and also between flocks in the same year. It was apparent that the time of the spring rise was related in some way to the time of lambing. Consider first, flock A. In 1950 lambing was late, most lambs being born in the first week of April. The spring rise occurred in mid-May. In 1951 about half the flock lambed in late February, but due to failure of a ram, the remainder of the lambing period was protracted and extended from the beginning of April to mid-May; the spring rise in this case showed a double peak, one in April and the other, a lower one extending from mid-May until the end of June. In 1952 the main lambing time was at the beginning of April, the main rise of egg counts was mid-April, although a second small peak occurred in mid-May. In 1953 the main lambing time was in February and the main peak was at the end of March. In flock B there was a similar correlation between lambing time and the time of the spring rise in egg counts.

In 1951 five ewes in flock A were selected for early mating. One was barren but the other four lambed in January. One died at lambing time. Samples were taken at weekly intervals before lambing and every two days after lambing. Other ewes in the flock were sampled with the same frequency and sampling continued until the end of June. This ensured that frequent samples were taken from ewes which lambed at different times during the period 1 January to 10 March. The time of maximum egg count is plotted against the time of lambing for each sheep (see Fig. 5).

On farm C in 1952 it was more difficult to obtain records of the actual day of lambing for each sheep but the shepherd marked the sheep with different colours according to the week in which they lambed. Samples were taken and the results are summarized in Fig. 4*b*. The time of rise of egg counts in the different groups followed the same sequence as the time of lambing of the groups and the intervals between the spring rises of different groups was approximately the same as the intervals between lambing.

(*c*) *Seasonal incidence of different species*

While much of the following data has been derived from differential larval counts, additional evidence has been obtained by means of the egg differentiation method described in a previous paper (Cunliffe & Crofton, 1953).

At no time was there only one species present, but the incidence of different species varied at different times of the year. The succession of species from the point of view of maximum numbers, was, with minor discrepancies, similar to that

described by Morgan and his co-workers (*op. cit.*) for hill pastures in Scotland. The discrepancies might be explained on the basis of differences of climatic conditions between the two regions. There appeared to be no significant difference between the Gloucestershire and Somerset farms except in the case of *Nematodirus*.

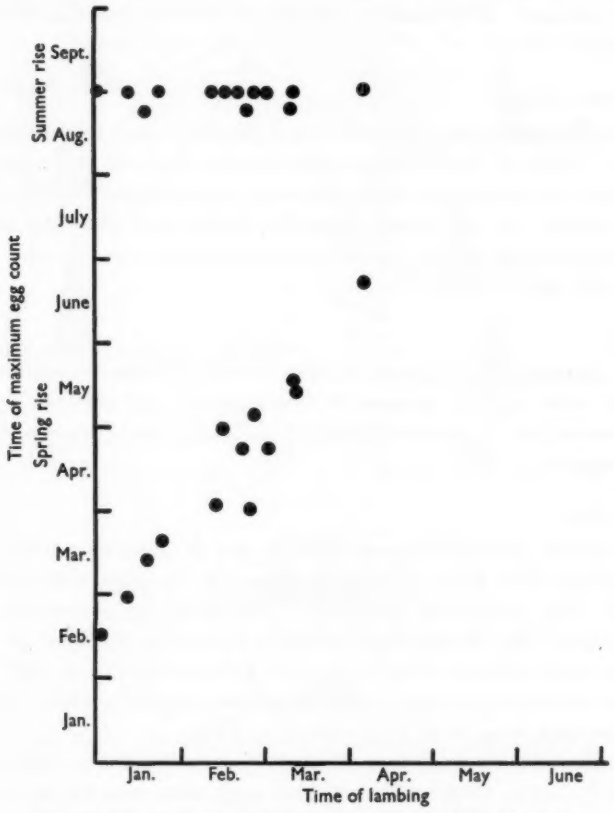

Fig. 5. The time of maximum egg count of individuals in spring and summer plotted against the time of lambing.

Haemonchus contortus

This was present in small numbers on all the farms, but was not at first detected in larval cultures. The egg differentiation method which is very sensitive for *Haemonchus* first betrayed its presence. Post-mortem examinations confirmed this, but very few worms were recovered from any individual sheep. Small numbers of eggs were detected throughout the year, most being found at the time of the spring rise.

Ostertagia spp.

These worms were most numerous in the spring and they contributed more to the spring rise than any other species. They were present at the time of the summer rise but were less important numerically than species of *Trichostrongylus*.

Trichostrongylus axei

The greatest numbers of this species occurred during the spring and summer rises, but numerically it was not the most important species at either of these times. Compared with *Ostertagia* spp. it was less important in spring but more important in summer. In summer it was less numerous than the other species of *Trichostrongylus*.

Trichostrongylus vitrinus

The records for this species might include *T. colubriformis*, but this latter species was found so rarely at post-morten examination that the intestinal species of *Trichostrongylus* are referred to as *T. vitrinus*. It was present in very small numbers in the spring, but was most numerous during and after the summer rise. During the summer rise it was slightly more numerous than *T. axei*. It was the predominant species in winter.

Cooperia spp.

Species of *Cooperia* were present in very small numbers throughout the year, but numbers were slightly greater in late summer and autumn. From post-mortem examinations *C. curticei* appeared to be the main species, *C. oncophora* occurring rarely.

Nematodirus spp.

The only species detected at post-mortem was *N. filicollis*. More information about this species than most of the others because the eggs were easily detected and recorded. Fig. 6*a–c* show the mean *Nematodirus* egg counts for flocks A, B and C respectively. The *Nematodirus* infection differed on these farms. On flock A the maximum mean number of eggs per gram was less than 24 in 1952. On flock B the maximum numbers were 59 in 1950, 56 in 1951 and 52 in 1952. On flock C the maximum numbers were 28 in 1951 and 31 in 1952.

In flock A the time of maximum numbers occurred in the months of June and July in 1950–2, but in 1953 no *Nematodirus* eggs were seen during these months and maximum numbers occurred during March and April. In each year, few or no *Nematodirus* eggs were found after July.

In flock B, where the number of *Nematodirus* eggs was much higher the greatest numbers occurred earlier than on flock A. The times of maxima are not known precisely because sampling in this flock was suspended during the lambing and the immediate post-lambing periods. Reference to counts on individual sheep shows that maximum counts occurred mainly in June 1950, April 1951, May and June 1952 and May 1953. It is interesting that the general pattern was closely related to the time of the spring rise. Few *Nematodirus* eggs were recorded after August in any of the years.

Strongyloides papillosus

This species occurred sporadically throughout the year but was most numerous in the autumn.

Other species

Bunostomum trigonocephalum, *Oesophagostomum venulosum*, *Chabertia ovina* and *Trichuris ovis* occurred in all flocks, but the numbers were too low to show any special trends.

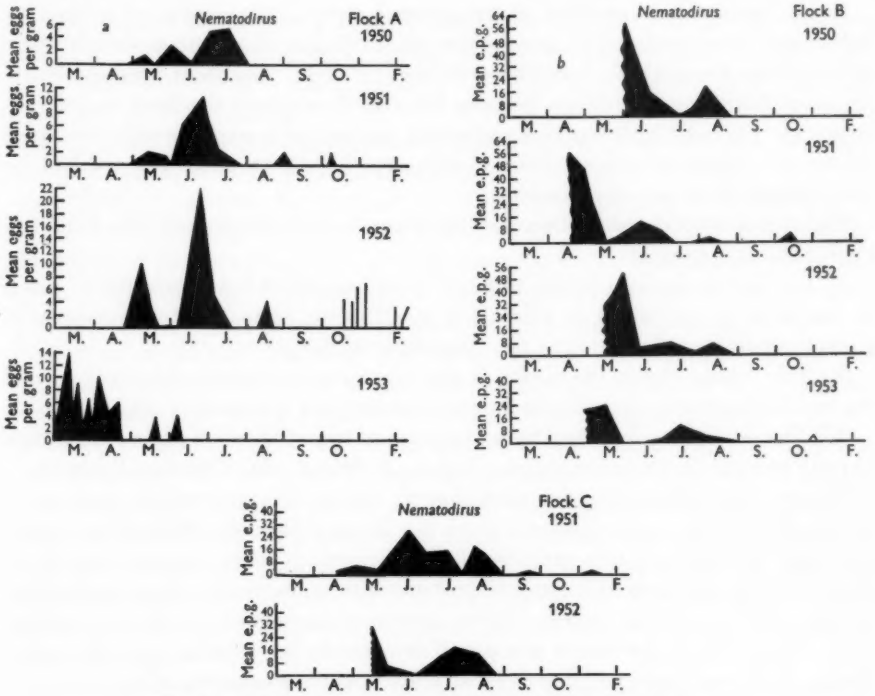

Fig. 6*a–c*. The mean number of *Nematodirus* eggs per gram for sheep in flocks A, B and C (1950–3).

IV. DISCUSSION

The degree of infection as measured by worm egg counts was lower in flock A than in flocks B and C. The rate of stocking of flock A was considerably lower than in the case of the other two flocks; in these latter the number of sheep per acre was approximately the same. In all three flocks, however, the general trend of egg counts was the same, although sheep in flock A received no anthelmintic treatment, while those in flock B were occasionally, and those in flock C repeatedly, dosed with phenothiazine and CuNic mixture. In all three flocks the counts were low for most of the year but there were two periods of increase: one, occurring mainly in April or May and referred to throughout as the spring rise; the other, in August, referred to as the summer rise.

The spring rise appears to be similar in most respects to that described by Morgan, Parnell and others (*op. cit.*) in Scotland, but, because of the relative infrequency of sampling imposed upon the Scottish workers by local conditions, it is

difficult to make detailed comparisons of the time of occurrence and duration of the increases in egg-count. Although there is some evidence of high counts in summer in some of the Scottish results, the intervals between sampling do not make it possible to decide whether or not there is a definite summer rise or merely a protracted period of high egg counts starting in spring.

In the spring rise described in the present work, examination of counts for individual sheep sampled at 2- or 3-day intervals has shown that the duration of the spring rise is short, usually less than a fortnight. The short duration is not apparent from grouped figures because the rise throughout the flock is not synchronous. Grouped figures give a misleading impression in another way: the mean figures are weighted by low counts of animals which have either not reached, or have passed their period of increase.

The spring rise has been discussed by a number of workers and the following explanations suggested:

(i) The spring rise is a direct result of newly acquired infections due to a fall in resistance of the sheep as a result of a fall in nutritional status, exposure to adverse weather and pregnancy (see Morgan *et al.* 1951).

(ii) The spring rise is the result of the maturing of worms which have overwintered as larvae in the mucosa of the intestine (see Naerland, 1949).

(iii) The spring rise is caused by an increase in egg production of mature worms already present in the intestine (see Cushnie & White, 1948; Naerland, 1949.)

Morgan *et al.* (1951), who concluded that the spring rise was produced by a newly acquired infection, quote Kauzal's work (see Ross & Gordon, 1936) which stated that eggs of *Ostertagia* spp. may develop and hatch at temperatures below 5° C. They deny on the basis of a series of post-mortem examinations that fourth stage larvae overwinter in the mucosa. Using data from the same series of observations they demonstrated that there was a real increase in the number of adult worms during April and June, and dismissed as irrelevant the suggestion of increased egg production of individual worms made by Cushnie & White (1948), Naerland (1949). The spring rise has been observed in the U.S.A. (Hawkins *et al.* 1944), Seghetti & Marsh, 1945, in Norway (Naerland, 1949) as well as in Scotland and England. It seems unlikely, therefore, that there is any basic difference between the spring rise in Scotland and that in south-west England when the phenomenon is so widespread.

The explanation given by Morgan and his co-workers cannot apply to the spring rise in south-west England without considerable modification. The reasons are:

(*a*) The spring rise is closely linked with the time of lambing and the spring rise can occur at different times from February to June in individuals in the same flock. This difference in timing is shown in the present work; and Morgan *et al.* (1951) say 'During these investigations [1948–50] it has been found that there is a tendency for the spring increase in worm egg output to occur earliest in the first ewes to lamb....'

(*b*) The onset of the rise is sudden and one might expect a decrease in the resistance of ewes as a result of the low nutritional status, exposure to adverse weather conditions and pregnancy to be gradual rather than sudden. Admittedly

it might be considered that there is a particular threshold value of resistance below which worms may develop. If this is accepted then if the number of infective stages available is higher in May than in February it might be expected that a late rise would be higher than an earlier one. This is not so. Observations have shown that although infective larvae occur on pastures in the South-West during winter, the number are very low. There are two reasons for the low numbers: first, the rate of development of eggs is low because of climatic conditions; secondly, the number of eggs passed on to the pasture is low. It is suggested, therefore, that the spring rise, which is of very short duration, and marked by a steep initial rise, cannot be due to a sudden breakdown of resistance because in the first 3 months of the year, at least, the number of infective stages available is not sufficient to account for the great increase. On the other hand, the steep initial rise seems to preclude a gradual increase of mature worms. Morgan *et al.* (1951) comment on the very large number of larvae to be found in the lumen of the abomasa of ewes during March and April. Their figures show that the numbers are greater than in any of the other months. In the present work more larvae have been found in the abomasa of ewes in spring than at any other time of the year. It is suggested that the number of larvae increases by a gradual accumulation over several weeks and not as a result of a sudden breakdown of resistance and immediate pick up, because in the earlier months of the year at least, the maximum rate of uptake of infective larvae, governed by the amount of pasture contamination, is too low to account for the large numbers of larvae being acquired almost simultaneously. If, however, acquisition of infective stages does not occur suddenly, then it is difficult to explain why there is not a gradual increase in the number of worm eggs passed by the ewes. The only explanation offered is that in the ewes the loss of resistance due to infection is gradual but progressive and that there are the following stages:

(i) Low intake of infective larvae, most of which if not all are eliminated without development. This is the *resistant state*.

(ii) Low intake of infective larvae but progressively more of these establish themselves. Full development is inhibited but the larvae are not eliminated. They do not necessarily invade and remain in the mucosa (see Threlkeld, 1934). This is the *accumulative stage* and represents the first stage in loss of resistance.

(iii) Low intake of infective larvae but many or most of these establish themselves and develop to maturity. The removal of inhibition of development to maturity permits the accumulated fourth and fifth stages to reach sexual maturity. Thus egg laying is simultaneous, although the worms have spent varying times within the host: This is the last stage in loss of resistance.

These stages are purely hypothetical but explain the observed facts. There is some hint of accumulation of larvae in the Scottish workers data. Further evidence for belief in these suggested stages is based upon the general observation that outbreaks in susceptible young animals, e.g. lambs, are usually preceded by an increase in worm egg counts, whereas in older animals outbreaks, presumably due to a loss of resistance cannot be *predicted* on the basis of egg counts.

So far, only the increase in egg counts has been considered, and indeed this has

been the main preoccupation of most previous workers. The rapid fall in the egg count is of equal interest. If we accept that the rise is due to a loss of resistance then we must also accept that the rapid recovery is due to an increase in resistance. The whole process, rise and fall of egg counts takes place 6–8 weeks after lambing. This includes the main lactation period, and it is well known that the supply of milk falls rapidly 6 weeks to 2 months after lambing. It is tempting to suggest that the fall of resistance is accelerated by the strain of milk production and that the resistance increases when the milk yield drops. This cannot be wholly accepted because the rise occurs in barren ewes and in ewes which abort or lose their lambs at birth. The occurrence of the spring rise in these cases suggests that the cause of loss of resistance is complex and possibly related to some basic rhythm of reproduction. The summer rise has not been remarked upon by other workers. It is of short duration and coincides with weaning time. There seems to be little evidence as to its cause and I am indebted to Dr E. L. Taylor for the suggestion that this short rise is not a real increase in egg production but a fall in the amount of faeces due to a temporary reduction of feeding which occurs as a result of disturbance and distress of ewes when they are separated from their lambs. This explanation is accepted because so far as has been observed, the summer rise does not occur in barren ewes.

The significance of the spring rise in the dynamics of the parasite population of a flock is obvious. The high rate of egg production some 6–8 weeks after lambing will provide a nucleus of infective stages available to the young and susceptible lambs. This must not be over-emphasized, however, because the short duration of the rise limits the total egg production, and the number of available infective stages is only enough to lay the seeds of infection in the lambs at their most susceptible stage. This will be discussed in a later paper.

SUMMARY

1. Details are given of worm egg counts carried out on faeces from ewes during 1950–3.

2. Two periods of high egg counts were recorded each year; one during spring and the other in late summer.

3. The first increase appeared to correspond to the spring rise of other workers. It has been shown to be of short duration in each individual ewe, and the time of its occurrence directly related to the time of lambing.

4. Details are given of the seasonal incidence of different species of nematodes.

5. The spring rise is discussed and a theory is advanced to account for the phenomenon. It is emphasized that the fall in egg counts after the rise is as important as the increase.

6. A suggestion that the summer rise is spurious and caused by a reduction in volume of faeces passed during the first week after weaning is accepted.

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THE BEHAVIOUR OF *TOXOPLASMA GONDII* ON SERUM-AGAR CULTURE

By R. J. V. PULVERTAFT, J. C. VALENTINE and W. F. LANE

The John Burford Carlill Pathological Laboratory,
Westminster School of Medicine, London

(With Plate XVI)

INTRODUCTION

Many parasites, including all known viruses, characteristically lead an intracellular existence, and are known as 'obligatory intracellular parasites'. It is, however, obvious that—whether the parasite is a virus, a bacterium, or a protozoon—transmission of infection from host to host, or from cell to cell within a single host, must involve an extracellular stage, however short in duration. This extracellular stage is, for the purpose of therapeutics of highest importance, since evidence accumulates that within the protecting walls of a cell parasites are relatively protected against chemotherapeutic agents and antibiotics, while susceptible to their action in the extracellular phase.

The extracellular stage of a parasite may in nature be exceedingly short. Each mammalian cell is in contact with other cells, and this is true even of the circulating blood cells, though possibly not of cells in lymphatic channels. But when, for example, a malaria parasite leaves an erythrocyte some period must elapse before it enters a new one, and some mechanism must exist whereby it gains ingress. Erythrocytes are not phagocytic; the emergent parasites after cell rupture are not motile. Authorities are silent on the question of cell to cell transference in malaria, although the diagrams in text-books beg the question by arrows indicating active migration.

One of us (R.J.V.P.) has watched human and murine malaria parasites developing in erythrocytes on serum agar, as described below; but has never observed invasion of an erythrocyte by an emergent parasite. In fact, the motionless parasite appears to be phagocytosed by polymorphs. In the case of *Babesia* the emergent parasites make a few spasmodic movements for minutes only; but they never have been seen to enter erythrocytes, or to be phagocytosed.

There is thus ample room for research into the mechanism of transference of parasites in this all-important group; the question can only be settled conclusively by direct observation. We have studied the problem in a peculiarly suitable parasite, *Toxoplasma gondii*. This parasite is large, readily kept alive in mice and fertile eggs, and survives well for adequate periods in tissue cultures. Finally, the method of translation from cell to cell has been both observed and recorded cinematographically.

MATERIAL AND METHODS

The technique used was basically slide culture on serum agar as previously used for the culture of mammalian cells (Pulvertaft, 1952; Pulvertaft & Jayne, 1953). All that is needed is the provision of solidified serum; and this can be done by adding 1 c.c. of 3% agar in distilled water to 3 c.c. of serum, and pouring the mixture into a 5 cm. Petri dish; small cubes are cut out, and placed on microscope slides. The cells to be examined are placed on the agar, a coverslip is applied, and the margins are sealed with wax. Cultures are examined at 37° C. for human cells, and 39° C. for murine cells, the microscope being mounted in a Perspex incubator. Phase contrast (Cooke Troughton and Simms) is used, and cinematographic records taken by time-lapse technique.

It is of course possible to polish and elaborate the above method to any degree that the observer's taste or conscience may dictate, but the simplest methods—any serum and any agar—will usually suffice in the case of *Toxoplasma*. The following notes may be useful to those anxious to elaborate the method.

(1) *Sterility*. This can almost always be ensured by adding 10 units/c.c. of penicillin. Contaminations are almost unknown.

(2) *Quality of agar*. A specially purified agar (Noble formula, B.D.H.) is available, as used for microbiological assay. We have used this throughout, but have not noted any disadvantage in standard bacteriological agars.

(3) *Nature of serum*. We have maintained in healthy life and in many cases in frequent mitosis, cells from a wide variety of animals on a wide variety of sera. Plasma is to be avoided as the platelets make the medium hazy. Light colour and complete translucency are desirable. The sera most readily obtained in a clinical laboratory are peritoneal effusions, and we select for choice those from cases of malignancy.

For murine cultures, however, an admirable medium is obtainable by injecting mice intraperitoneally with one of the 'ascites' tumours; Krebs no. 2 or S. 37 induce extreme ascites. These fluids clot spontaneously thus removing cells, and are optimal for murine cells.

All sera on keeping either at +4° C. or −40° C. lose O_2, CO_2 and glucose; and the amino-acid content cannot be taken for granted. Thus the pH of preserved serum may be relatively unsuitable. For optimal results we have added 0·20% of glucose, and 0·2% of Casamino acids. (Difico) to the serum; and 95% O_2 with 5% CO_2 have been bubbled through the serum until the pH is 7·4. On such a medium, bone marrows, lymph glands, malignant cells and histiocytes survive excellently, but we would emphasize that these precautions are quite unnecessary to study the behaviour of *Toxoplasma*. In the experiments described the serum was diluted 50% with Tyrode's solution; but recent work has shown that the only effect of the Tyrode is to adjust the pH of the medium and to add glucose. Anyone who has worked with Tyrode's solution will avoid it if possible.

The purpose of the serum agar is twofold. In the first place cells between two glass surfaces, slide and cover-slip are easily ruptured if pressure is applied, or are too thick and condensed for examination if pressure is light. Secondly, cells very

soon die under these conditions; in our experience mammalian cells rarely survive more than 2 hr. at 37° C. and degenerate rapidly.

The agar supplies a yielding surface, on which cells can be flattened to any required degree without rupture, although it is found that a gap of approximately 30μ exists between cover-slip and agar even when pressure is applied. Secondly, the agar supplies oxygen, glucose, amino-acids and all necessary electrolytes. Thus bone marrow may be kept alive for 3 weeks and lymphocytes for 5 days; many other cells have survived for over a month.

It may be added that a very wide variety of embryo extracts, tissue extracts, vitamins, amniotic fluids, yeast extracts, etc., have been added to serum without any obvious improvement; and we emphasize again that any serum and any agar in manageable proportions are quite satisfactory. It will be noted that the culture medium is slightly hypotonic. There is no advantage, however, in adding electrolytes to adjust this; indeed we have found that on the contrary better results often follow if small amounts of distilled water are added to the serum, for example 0·5 c.c. of distilled water to 2·5 c.c. of serum.

Toxoplasma gondii was first cultivated *in vitro* by Sabin & Olitsky (1937) using Li-Rivers minced chick embryo medium. Lock (1953) recorded its culture in embryo rat heart; no record has been found of its culture *in vitro* in adult cells, nor has the method of intercellular migration being recorded. Most authorities state that it is non-motile, but Guimarães & Meyer (1942) clearly described an extracellular motile phase in tissue culture, and with their account we are entirely in agreement.

The strain used was the first and indeed only strain hitherto isolated from a human case in this country and recorded by Valentine, Lane, Beattie & Beverley (1953). It was maintained in fertile hen's eggs, and was transferred to the peritoneum of a mouse. It was examined *in vitro* in the following cells: (1) macrophages from the peritoneum of a mouse; (2) mouse bone marrow, which is readily obtained by cutting across a femur, and aspirating the marrow with an exceedingly finely drawn glass pipette; (3) human bone marrow, taken into heparinized serum, whence particulate masses were selected; (4) human lymph gland: specimens from gastrectomies were selected; pulp was scraped with a scalpel; (5) leucocytes from rabbit; (6) mouse lung; infected lungs were minced with a razor blade, and the pulp placed on the agar blocks; (7) S 37 cells (mouse ascites tumour).

The inoculum of *Toxoplasma* was almost always obtained from the peritoneal exudate of an infected mouse, in which parasites are readily found, 24 hr. after inoculation, but in 7 days there is a rich exudate which swarms with them. It should be remembered that laboratory infection of man has been recorded and the infected animals and cultures should be treated with reasonable caution.

Essentially our experiments involve the transference of the parasite from the infected murine cells to other cells of other animals. The infected peritoneal exudate is therefore mixed on the agar block with the cells of the new host. This often involves cannibalism of the cells of one animal by those of another, e.g. human polymorphs ingest mouse lymphocytes.

Before describing the results it may be stated as axiomatic that a parasite can

only gain ingress to a cell by two mechanisms, one active and the other passive. It may penetrate a cell actively as a spermatozoon enters an ovum; or it may be ingested passively as a staphylococcus is ingested by a polymorph. Our results indicate that in the case of *Toxoplasma* both mechanisms can be observed.

RESULTS

Morphology

By phase contrast the intracellular parasite appears as a boat-shaped organism, $8 \times 3\mu$; when extruded it quickly shrinks in all dimensions. One end (the end which leads in the motile phase) is sharper than the other. The central portion is notably paler, and appears to be the nucleus; there is also a faint central karyosome. Both ends are filled with fine dark granules, which also extend along one side of the nucleus. A prominent translucent vacuole is usually seen at one end, rarely at both; one granule is usually far larger than the others.

It may be worth while to define what is meant by 'dark' and 'light' in phase contrast. Any object or granule viewed by phase contrast may be made to appear 'dark' or 'light' by alteration of the focus; moreover, since the depth of focus of a $\times 95$ lens is only 2μ, and the depth of a cell 30μ or more, each focus of a cell is an optical section. Thus identical granules and vacuoles appear light and dark at different focal planes, of which there are 15 per cell with a $\times 95$ lens. The following simple rule, however, determines whether a granule is to be described as 'light' or 'dark' by positive phase contrast.

Any small granule viewed by positive phase contrast may appear light or dark according to the adjustment of the focus. When in exact focus a 'dark' object looks dark; above and below the focal plane it looks 'light'. When in exact focus a 'light' object looks 'light'; above and below the focal plane it looks 'dark'. Therefore, the correct optical appearance is presented once only on varying the focus through the focal plane; the incorrect appearance twice. In its simplest form therefore the rule is that the appearance seen once only on varying the focus is the correct appearance.

Some modern phase-contrast equipment provides an infinitely variable contrast, and without doubt optical analysis of particulate cytoplasmic inclusions with this equipment will become a research procedure. The rule enunciated above holds good, however, for all equipment in standard use at present in this country.

The parasite never moves inside a cell unless the cell is itself active, when it is turned around with the cytoplasmic organoids. It is quite rigid, and is never deformed whatever shape the cell assumes. When the host cell contracts the parasite rests in a nuclear concavity. When a cell characteristically moves with its organoids grouped in the trailing portion of its cytoplasm, as does the lymphocyte, the parasite also is trailed behind.

It will be noted that all the cells invaded were motile, either actively, like leucocytes, or sluggishly, like S 37 cells. However, this is not significant, since no consistent success was obtained in attempts to keep epithelial cells alive by the agar technique.

Division of parasite

Parasites multiply slowly, occupying many hours in the process; and since the host cells are all motile, it is a laborious business to study division. Parasites were never seen to unite; multiplication was always asexual. The nucleus and organoids of the parasite are first duplicated, and the parasite becomes oval. It then undergoes fission, the sharp end separating first, and assumes a butterfly appearance, with the twins united posteriorly. Eventually the two parasites are separated and apposed as mirror image cells. No parasite ever formed more than two daughter cells.

Rupture of host cell

When exudates rich in parasites are mounted over agar, host cells are readily ruptured by pressing gently on the cover-slip with a needle while watching the cells, and most of the cinematographic and other studies were so obtained. Natural rupture was however often observed. The host cell, filled with parasites, becomes spherical and the parasites often assume a radial arrangement. The host cell often acquires a relatively rapid and oscillating rotary movement before rupture; it is not easy to understand how a spherical cell rotates on its axis without a visible mechanism of rotation, but many cells have this property on serum agar.

When the cell ruptures, the parasites are passively expelled, as from a dehiscent fruit, and are at first quite motionless. They then move in all directions, exhibiting movements of translation over distances up to 200μ and of deformation affecting only the sharp end, which is always the leading end. This is moved like the trunk of an elephant, in all directions, but chiefly upwards to the cover-slip. The parasite never rotates, since the organoids always occupy the same relative positions. Movement is sinuous and intermittent, and proceeds in porpoise-like undulations, with periods of rest progressively longer until, usually after 15 min., they come finally to rest; motion was never observed after 45 min.

The method of propulsion cannot be determined. There is no pseudopodal deformation of outline, no flagella or cilia are seen by dark-ground illumination, or are to be inferred by disturbance of environmental particles, nor is vermicular distortion of outline observed. Chemotaxis is excluded as sole agent, for motion is observed when no cells are anywhere near; but chemotaxis undoubtedly exists as the parasites clearly move to new host cells within reach. The most likely mechanism of propulsion is by surface tension forces, such as are exhibited by toy boats operated by camphor at the stern. Indeed in some cases a thin thread is seen to proceed from the posterior end of the parasite as it progresses apparently by jet propulsion.

The conditions of this experiment are highly abnormal; in its ordinary life cycle a parasite presumably finds its new host cell immediately adjacent, and in all dimensions of space. It is indeed surprising that it can move so far and for so long as it can. If a new host cell lies within 50μ of a parasite, it is approached in one movement; if further in a series of bounds. The host cell is always entered from below, and the parasite appears to select a point at the circumference for invasion, applying itself to the surface of the cell, and sliding around until it reaches the selected point, which appears to be that part of the cytoplasm related to the under-

lying concavity of nuclei which are reniform, as many are. More than one parasite has been observed to approach a single cell at the same point in the cell. The parasite creeps under the cell, turning as it does so at right angles to the tangent at the point of approach. As it proceeds under its outline is deformed continuously at the edge of the host cell. As soon as it is all beneath the cell it turns again through 90°, and lies alongside the nucleus filling the reniform concavity of the nucleus. It never again moves independently of the host cell. It is surprising that the invading host cell never ruptures at the time of invasion. This may be in some way related to the fact that the cell is always invaded from below, so that the perforation is sealed by the agar surface.

It would be wrong to dogmatize on the basis of these observations, prolonged though they have been, as to the fate of invaded cells; but our evidence is that after a period of a few minutes or several hours they suddenly assume violent activity, and often rupture, and the parasite enters another cell. Alternatively, the parasite is extruded in an investing layer of cytoplasm while the host cell, after further violent movements, dies. The evidence suggests that in the latter case both host cell and parasite die, since parasites invested with cytoplasmic cloaks did not multiply and were not phagocytosed. On the other hand, our evidence suggests that host cells actively invaded die, and that the parasite does not multiply in cells which it actively invades.

There remains the second method of gaining ingress to a host cell, by phagocytosis. The non-phagocytic lymphocyte is a favourite host cell of the parasite; but most of the other cells in which parasites have been seen are phagocytic. This includes the malignant ascites cell S. 37. We have not observed parasitism of nucleated red cells in bone marrow.

Phagocytosis of motionless parasites is very easily observed, and cinematographic records have been made. There seems to be no limit to the number of parasites which one cell will absorb; and there seems to be no toxic effect on the host cell following phagocytosis. This is a marked distinction between the effect of active and passive invasion of host cells; moreover, so far as our observations go, it is the phagocytosed parasite which divides.

Effects on mitosis of host cells

A surprisingly large number of invaded cells are found to be in mitosis or multinucleated. This might be because invasion decelerates the mitotic cycle; but in fact this is not so, as mitosis continues speedily under the microscope. However, quite often it is interrupted by rupture of the cell; and we have never observed an invaded cell to divide normally. Usually, just as two nuclei are formed the parasites remain at one end of the still united cells, leaving the other free of parasites; the nuclei divide, but the cytoplasm does not, and a binucleated parasitized cell is formed. Such cells are very common. Thus either the parasite has an affinity for cells in mitosis, or it stimulates this process. The second suggestion is of considerable interest in view of the possible relationship between intracellular parasites and neoplasia—cf. the Rous Sarcoma and in general the virus theory of malignancy.

DISCUSSION

These experiments indicate that this obligatory parasite gains ingress to host cells in two different ways, and suggests that the subsequent fate of host and parasite may be dependent on which mechanism is involved; it seems clear that active invasion is lethal to the host cell, and it probable that reproduction of the parasite is confined to those passively ingested. There is some, if far from conclusive, evidence for a two-phase life cycle of the parasite, the actively invasive toxic phase, and the passively ingested reproductive phase. It is also suggested that invasion of the host cell may affect its process of reproduction in certain cases, leading to the formation of multinucleated cells, and perhaps stimulating host cell reproduction.

Preliminary tests with other intracellular parasites, such as *Plasmodium*, *Babesia* and *Leishmania*, indicate that there is a great deal yet to be known as to their life cycle. The method here followed, whereby invasion of host cells can be watched and where the host cells of one animal may be used to infect the host cells of a different species, might be profitably employed in the study of other parasites.

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EXPLANATION OF PLATE XVI

Fig. 1. Cell from murine peritoneal exudate, Sarcoma 37, showing all stages of reproduction of parasite. (× 2500).
Fig. 2. Murine peritoneal exudate. Rosette formation of parasites. (× 2500).
Figs. 3–5. Murine bone marrow. Invasion of cell in mitosis; partition of parasites; formation of bi-nucleated cell. (× 1040).
Fig. 6. Murine bone marrow. Rupture of murine peritoneal cell and invasion of murine bone marrow cell. (× 1040).

(All illustrations are of living serum agar preparations, phase contrast illumination; temperature 39° C.)

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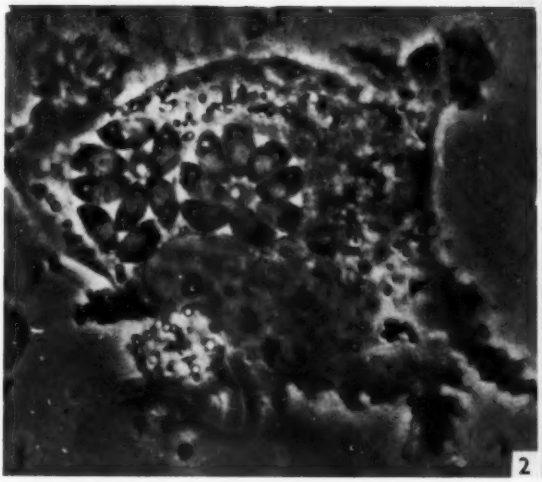
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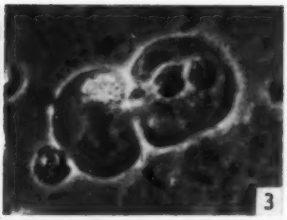
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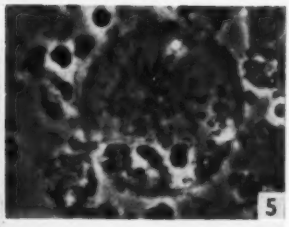
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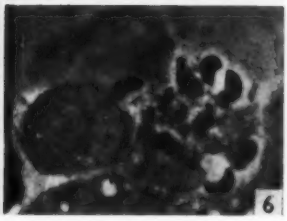
6

INDEX OF AUTHORS

BISHOP, ANN. The effect of sulphadiazine, proguanil and 2:4-diamino-6:7-di*iso*propyl-pteridine upon gametocyte production in *Plasmodium gallinaceum* (Brumpt, 1935), 120

BISHOP, ANN. The action of 2:4-diamino-6:7-di*iso*propylpteridine upon *Plasmodium gallinaceum* and its relation to other compounds which are pteroylglutamic acid antagonists. (With 1 Figure in the Text), 450

BROWNING, T. O. On the structure of the spiracle of the tick, *Ornithodoros moubata* Murray. (With 1 Figure in the Text), 310

CORBETT, M. PAULINE, *see* GRESSON, R. A. R.

CROFTON, H. D. The ecology of the immature phases of trichostrongyle parasites. V. The estimation of pasture infestation. (With 3 Figures in the Text), 313

CROFTON, H. D. Nematode parasite populations in sheep on lowland farms. I. Worm egg counts in ewes. (With 6 Figures in the Text), 465

DAVIES, A. MICHAEL, *see* ELIAKIM, M.

DE MELLO, I. FROILANO. *Pseudotrichonympha sertaneja* n.sp. (Protozoa, Mastigophora), from the intestine of a new termite (*Rugitermes* sp.) collected in Brazil. (With 3 Figures in the Text), 24

DE MELLO, I. FROILANO. On a new species of *Stephanonympha* (Protozoa, Mastigophora) from the intestine of the Brazilian termite, *Neotermes hirtellus*. (With Plate VI), 30

DEORAS, P. J. Breeding the Indian house-fly (*Musca domestica nebulo* Fabr.) for experimental studies. (With 5 Figures in the Text), 304

DINNIK, J. A. *Paramphistomum sukari* n.sp. from Kenya cattle and its intermediate host. (With 7 Figures in the Text), 414

DINNIK, J. A. and DINNIK, N. N. The life cycle of *Paramphistomum microbothrium* Fischoeder, 1901 (Trematoda, Paramphistomidae). (With 6 Figures in the Text), 285

DINNIK, N. N., *see* DINNIK, J. A.

DRAPER, C. C. The duration of residual immunity following spontaneous cure of *Plasmodium berghei* in rats. (With 1 Figure in the Text), 338

ELIAKIM, M. and DAVIES, A. MICHAEL. The complement-fixation test in bilharziasis. I. The value of different extracts of *Schistosoma mansoni* and *Fasciola hepatica* worms as antigens. (With 2 Figures in the Text), 407

FAHMY, M. A. M. An investigation on the life cycle of *Trichuris muris*. (With 7 Figures in the Text), 50

FYFE, M. L. *Tricotyledonia genypteri* n.g., n.sp., a three-suckered trematode from the red ling, *Genypterus blacodes* Bloch & Schn. (With 5 Figures in the Text), 325

GNANAMUTHU, C. P. *Choniosphaera indica*, a copepod parasitic on the crab *Neptunus* sp. (With 29 Figures in the Text), 371

GOTTO, R. V. *Lichomolgides cuanensis* n.g., n.sp., an ascidicolous copepod occurring in *Trididemnum tenerum* (Verrill). (With 20 Figures in the Text), 379

GRESSON, R. A. R. and CORBETT, M. PAULINE. A morphological study of a fish tapeworm, *Proteocephalus pollanicola*. (With 7 Figures in the Text), 34

HAIBA, M. H. The pH of the alimentary tract in normal and *Giardia*-infected culture mice. (With 2 Figures in the Text), 387

HISCOCK, IAN D. A new species of *Otobothrium* (Cestoda, Trypanorhyncha) from Australian fishes. (With 6 Figures in the Text), 65

HOPKINS, SEWELL H. The American species of trematode confused with *Bucephalus* (*Bucephalopsis*) *haimeanus*. (With 13 Figures in the Text), 353

HOY, J. M. The biology and host range of *Neoaplectana leucaniae*, a new species of insect-parasitic nematode. (With 5 Figures in the Text), 392

KULASIRI, CHARLES. Some cestodes of the rat, *Rattus rattus* Linnaeus, of Ceylon and their epidemiological significance for man, 349

LANE, W. F., *see* PULVERTAFT, R. J. V.
LLEWELLYN, J. Observations on the food and the gut pigment of the Polyopisthocotylea (Trematoda: Monogenea). (With Plate XV), 428

MCCONNACHIE, ELSPETH W. The action of amoebicidal drugs on *Entamoeba invadens* Rodhain, 1934 *in vitro*, 132
MCCONNACHIE, ELSPETH W. The influence of environmental factors on the size of the cysts of *Entamoeba invadens* Rodhain, 1934. (With 3 Figures in the Text), 342
MEYER, HERTHA and PORTER, KEITH R. A study of *Trypanosoma cruzi* with the electron microscope. (With Plates I–V), 16
MORIYA, SHOOJI. The reliability of the current diagnostic methods for the identification of helminth eggs. (With 1 Figure in the Text), 300
MOSCONA, A., *see* THEODOR, O.

NEAL, R. A. Amoebae found in the intestine of lizards from the Sudan. (With 9 Figures in the Text), 422

PORTER, KEITH R., *see* MEYER, HERTHA
PULVERTAFT, R. J. V., VALENTINE, J. C. and LANE, W. F. The behaviour of *Toxoplasma gondii* on serum-agar culture.

RADFORD, CHARLES D. The larval genera and species of 'harvest mites' (Acarina: Trombiculidae). (With 108 Figures in the Text), 247
ROBERTS, O. JOY. The effect of cortisone on *Plasmodium berghei* infections. (With 4 Figures in the Text), 58
ROBERTS, O. JOY. The effect of cortisone on *Plasmodium berghei* infections. (With 6 Figures in the Text), 438

SINHA, T. B. New species of larval mites from India (Acarina: Trombiculidae). (With 4 Figures in the Text), 329
SMIT, F. G. A. M. New bat-fleas (Siphonaptera: Ischnopsyllidae). (With 32 Figures in the Text), 144
SMITH, KENNETH M. and XEROS, N. Electron and light microscope studies of the development of the virus rods of insect polyhedroses. (With Plates VII–XII), 71
SMITH, KENNETH M. and XEROS, N. A comparative study of different types of viruses and their capsules in the polyhedroses and granuloses of insects. (With Plates XIII and XIV), 400

TATE, P. Notes upon the biology and morphology of the immature stages of *Neottiophilium praeustum* (Meigen, 1926) (Diptera: Neottiophilidae) parasitic on birds. (With 11 Figures in the Text), 111
THEODOR, O. and MOSCONA, A. On the bat parasites in Palestine. I. Nycteribiidae, Streblidae, Hemiptera, Siphonaptera. (With 105 Figures in the Text), 157
THURSTON, JUNE P. Anaemia in mice caused by *Eperythrozoon coccoides* (Schilling 1928). (With 11 Figures in the Text), 81
THURSTON, JUNE P. The chemotherapy of *Plasmodium berghei*. II. Antagonism of the action of drugs, 99

ULLMAN, H. Observations on a new cercaria developing in *Melanopsis praemorsa* in Israel. (With 20 Figures in the Text), 1

VALENTINE, J. C., *see* PULVERTAFT, R. J. V.

WERTHEIM, GUTA. A new anophlocephalid cestode from the gerbil. (With 3 Figures in the Text), 446
WILSON, S. G. *Rhipicephalus hurti* n.sp. (Ixodidae) from Kenya game and domestic animals. (With 6 Figures in the Text), 277

XEROS, N., *see* SMITH, KENNETH M.
XEROS, N., *see* SMITH, KENNETH M.

INDEX OF SUBJECTS

Amoebae found in the intestine of lizards from the Sudan, 423
Amoebicidal drugs, the action of, on *Entamoeba invadens* Rodhain, 1934, 132

Bat-fleas (Siphonaptera: *Ischnopsyllidae*), 144
Bat parasites in Palestine, 157
Bilharziasis, the complement-fixation test in, 407
Bucephalus (*Bucephalopsis*) *haimeanus*, the American species of trematode confused with, 363

Cercaria, observations on, in *Melanopsis praemorsa* in Israel, 1
Choniosphaera indica, a copepod parasitic on the crab *Neptunus* sp., 371
Comparative study of different types of viruses and their capsules in the polyhedroses and granuloses of insects, 400
Cortisone, the effect of, on *Plasmodium berghei* infections, 58, 438

Electron and light microscope studies of the development of the virus rods of insect polyhedroses, 71
Entamoeba invadens Rodhain, 1934, the action of amoebicidal drugs on, 132
Entamoeba invadens the influence of environmental factors on the size of the cysts of, 342
Eperythrozoon coccoides (Schilling, 1928), anaemia in mice caused by, 81

Gametocyte production, in *Plasmodium gallinaceum*, the effect of sulphadiazine, proguanil and 2:4-diamino-6:7-di*iso*propylpteridine upon, 120
Genypterus blacodes Bloch & Schn., the red ling, *Tricotyledonia genypteri* n.g., n.sp., a three-suckered trematode from, 325
Gerbil, a new anoplocephalid cestode from the, 446
Giardia-infected culture mice, the pH of the alimentary tract in, 387

'Harvest mites' (Acarina: *Trombiculidae*) the larval genera and species of, 247
Helminth eggs, reliability of the current diagnostic methods for the identification of, 300

Indian house-fly (*Musca domestica nebulo* Fabr.) breeding for experimental studies, 304
Insect polyhedroses, electron and light microscope studies of the development of the virus rods of, 71
Insect polyhedroses and granuloses, a comparative study of different types of viruses and their capsules in, 400
Ischnopsyllidae, new bat-fleas, 145

Larval mites from India (Acarina: *Trombiculidae*) new species of, 329
Lizards, from the Sudan, amoebae found in the intestine of, 423
Lichomolgides cuanensis n.g., n.sp., an ascidicolous copepod occurring in *Trididemnum tenerum* (Verrill), 379

Melanopsis praemora, observations on a new *Cercaria* developing in, in Israel, 1
Mice, anaemia in, caused by *Eperythrozoon coccoides* (Schilling, 1928), 81
Mice, giardia-infected, the pH of the alimentary tract in, 387
Monogenea, observations on the food and the gut pigment of the *Polyopisthocotylea*, 428
Musca domestica nebulo Fabr., the Indian house-fly, breeding for experimental studies, 304

Nematode parasite populations in sheep on lowland farms, 465
Neoaplectana leucaniae, a new species of insect-parasitic nematode, the biology and host range of, 393
Neotermes hirtellus, from the intestine of, a new species of *Stephanonympha*, 30
Neottiophilum praeustum (Meigen, 1826), notes upon the biology and morphology of the immature stages of, parasitic on birds, 111
Neptunus sp., *Choniosphaera indica*, a copepod parasitic on, 371

Ornithodoros moubata Murray, the tick, on the structure of the spiracle of, 310
Otobothrium (Cestoda, Trypanorhyncha), a new species of, from Australian fishes, 65

Paramphistomum microbothrium Fischoeder, 1901 (Trematoda, Paramphistomidae) the life cycle of, 285
Paramphistomum sukari n.sp., from Kenya cattle and its intermediate host, 414
Plasmodium berghei infections, the effect of cortisone on, 58, 438
Plasmodium berghei, the chemotherapy of, 99
Plasmodium berghei, the duration of residual immunity following spontaneous cure of, in rats, 338
Plasmodium gallinaceum (Brumpt, 1935), the effect of sulphadiazine, proguanil and 2:4-diamino-6:7-di*iso*propylpteridine upon gametocyte production in, 120
Plasmodium gallinaceum, the action of 2 : 4-diamino-6 : 7-di*iso*propylpteridine upon, and its relation to other compounds which are pteroylglumatic acid antagonists, 450
Polyopisthocotylea (Trematoda: Monogenea) observations on the food and the gut pigment of, 428
Proteocephalus pollanicola, a morphological study of a fish tapeworm, 34
Pseudotrichonympha sertaneja n.sp. (Protozoa, Mastigophora), from the intestine of a new termite (*Rugitermes* sp.) collected in Brazil, 24

Rats, spontaneous cure of *Plasmodium berghei* in, 338
Rattus rattus Linnaeus, some cestodes of, and their epidemiological significance for man, 349
Rhipicephalus hurti n.sp. (Ixodidae) from Kenya game and domestic animals, 277
Rugitermes sp., *Pseudotrichonympha sertaneja* n.sp., from the intestine of, collected in Brazil, 24

Sheep, nematode parasite populations in, 465
Stephanonympha (Protozoa, Mastigophora), on a new species of, from the intestine of the Brazilian termite, *Neotermes hirtellus*, 30

Toxoplasma gondii, the behaviour of, on serum-agar culture, 478
Trichostrongyle parasites, the ecology of the immature phases of, 313
Trichuris muris, an investigation on the life cycle of, 50
Tricotyledonia genypteri n.g., n.sp., a three-suckered trematode from the red ling, *Genypterus blacodes* Bloch and Schn., 325
Tridemnum tenerum (Verrill), *Lichomolgides cuanensis* n.g., n.sp., an ascidicolous copepod occurring in, 379
Trombiculidae, new species of larval mites from India, 329
Trombiculidae, the larval genera and species of 'Harvest mites', 247
Trypanosoma cruzi, a study of, with the electron microscope, 16

PAGE

DINNIK, J. A. *Paramphistomum sukari* n.sp. from Kenya cattle and its intermediate host. (With 7 Figures in the Text) 414

NEAL, R. A. Amoebae found in the intestine of lizards from the Sudan. (With 9 Figures in the Text) 422

LLEWELLYN, J. Observations on the food and the gut pigment of the Polyopisthocotylea (Trematoda: Monogenea). (With Plate XV) 428

ROBERTS, O. JOY. The effect of cortisone on *Plasmodium berghei* infections. (With 6 Figures in the Text) 438

WERTHEIM, GUTA. A new anophlocephalid cestode from the gerbil. (With 3 Figures in the Text) 446

BISHOP, ANN. The action of 2:4-diamino-6:7-di*iso*propylpteridine upon *Plasmodium gallinaceum* and its relation to other compounds which are pteroylglutamic acid antagonists. (With 1 Figure in the Text) 450

CROFTON, H. D. Nematode parasite populations in sheep on lowland farms. I. Worm egg counts in ewes. (With 6 Figures in the Text) 465

PULVERTAFT, R. J. V., VALENTINE, J. C. and LANE, W. F. The behaviour of *Toxoplasma gondii* on serum-agar culture 478

Index of Authors 485

Index of Subjects 487

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CONTENTS

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PAGE

RADFORD, CHARLES D. The larval genera and species of 'harvest mites' (Acarina: Trombiculidae). (With 108 Figures in the Text) 247

WILSON, S. G. *Rhipicephalus hurti* n.sp. (Ixodidae) from Kenya game and domestic animals. (With 6 Figures in the Text) 277

DINNIK, J. A. and DINNIK, N. N. The life cycle of *Paramphistomum microbothrium* Fischoeder, 1901 (Trematoda, Paramphistomidae). (With 6 Figures in the Text) 285

MORIYA, SHOOJI. The reliability of the current diagnostic methods for the identification of helminth eggs. (With 1 Figure in the Text) 300

DEORAS, P. J. Breeding the Indian house-fly (*Musca domestica nebulo* Fabr.) for experimental studies. (With 5 Figures in the Text) 304

BROWNING, T. O. On the structure of the spiracle of the tick, *Ornithodorus moubata* Murray. (With 1 Figure in the Text) 310

CROFTON, H. D. The ecology of the immature phases of trichostrongyle parasites. V. The estimation of pasture infestation. (With 3 Figures in the Text) . . 313

FYFE, M. L. *Tricotyledonia genypteri* n.g., n.sp., a three-suckered trematode from the red ling, *Genypterus blacodes* Bloch & Schn. (With 5 Figures in the Text) . 325

SINHA, T. B. New species of larval mites from India (Acarina: Trombiculidae). (With 4 Figures in the Text) 329

DRAPER, C. C. The duration of residual immunity following spontaneous cure of *Plasmodium berghei* in rats. (With 1 Figure in the Text) 338

MCCONNACHIE, ELSPETH W. The influence of environmental factors on the size of the cysts of *Entamoeba invadens* Rodhain, 1934. (With 3 Figures in the Text). 342

KULASIRI, CHARLES. Some cestodes of the rat, *Rattus rattus* Linnaeus, of Ceylon and their epidemiological significance for man 349

HOPKINS, SEWELL H. The American species of trematode confused with *Bucephalus* (*Bucephalopsis*) *haimeanus*. (With 13 Figures in the Text) 353

GNANAMUTHU, C. P. *Choniosphaera indica*, a copepod parasitic on the crab *Neptunus* sp. (With 29 Figures in the Text) 371

GOTTO, R. V. *Lichomolgides cuanensis* n.g., n.sp., an ascidicolous copepod occurring in *Trididemnum tenerum* (Verrill). (With 20 Figures in the Text) 379

HAIBA, M. H. The pH of the alimentary tract in normal and *Giardia*-infected culture mice. (With 2 Figures in the Text) 387

HOY, J. M. The biology and host range of *Neoaplectana leucaniae*, a new species of insect-parasitic nematode. (With 5 Figures in the Text) 392

SMITH, KENNETH M. and XEROS, N. A comparative study of different types of viruses and their capsules in the polyhedroses and granuloses of insects. (With Plates XIII and XIV) 400

ELIAKIM, M. and DAVIES, A. MICHAEL. The complement-fixation test in bilharziasis. I. The value of different extracts of *Schistosoma mansoni* and *Fasciola hepatica* worms as antigens. (With 2 Figures in the Text) 407

(*continued on inside of back cover*)

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